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THE IDENTIFICATION OF PRODIGIOSIN AND SIMILAR COMPOUNDS

by

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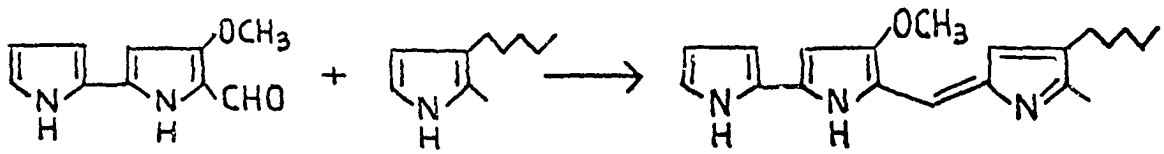
INTRODUCTION

The microorganism Serratia marcescens normally produces the red pyrryldipyrrylmethene pigment, prodigiosin. Final step in the biosynthesis of the pigment involves coupling of a bipyrrole, 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde, and a monopyrrole, 2-methyl-3-amylpyrrole (26).



Abnormally pigmented mutants of this organism produced by ultraviolet irradiation (13, 20, 29) can be divided into two classes: those which have a metabolic block in the pathway leading to the bipyrrole and those which are blocked in the synthesis of the monopyrrole. Certain mutants have the ability to induce formation of red pigment in other normally nonpigmented mutants. On the basis of observed patterns of syntrophic pigment production, mutants have been scored as either donor or receptor mutants and placed in a sequence reflecting the probable relative positions of their metabolic blocks. Three mutants in the bipyrrole pathway have been arranged in such a sequence: the orange mutant OF is a donor to colorless mutants H-462 and WCF, H-462 is a receptor to OF and a donor to WCF, and WCF is a receptor to both of the other two. Hence the sequence of steps blocked in these three mutants is most

probably: WCF → H-462 → OF. To verify this sequence it would be necessary to isolate the syntrophic pigment produced in each case and to demonstrate unequivocally that it is prodigiosin.

On the other hand, colorless mutant 9-3-3 accumulates the final bipyrrole precursor but is blocked in the pathway leading to the monopyrrole. When this strain is supplied with synthetic 2-methyl-3-amylpyrrole normal pigmentation ensues rapidly. It has been shown (28) that synthetic 2,4-dimethylpyrrole can be substituted for 2-methyl-3-amylpyrrole to yield an analog of prodigiosin differing from the wild-type pigment only in the alkyl substituents on the monopyrrole moiety of the molecule. It would be of interest to study the extent of substitution possible in the final coupling reaction, and to examine the properties of prodigiosin analogs so produced.

Mutant 9-3-3, however, has also been shown to produce a purple pigment when grown without addition of a monopyrrole but also without inorganic phosphate in the medium (29). Identification of this abnormal pigment should shed light on pyrrole metabolism in Serratia, particularly if it should turn out to be a compound closely related to prodigiosin but with an incomplete monopyrrole moiety. Furthermore, prodigiosin and prodigiosin-like compounds have been reported in microorganisms other than Serratia marcescens. Certain strains of Streptomyces and an unidentified marine bacterium have yielded prodigiosin-like compounds as reported by a variety of workers.

The purpose of the present investigation was to study prodigiosin and closely related compounds to establish analytical criteria for identification of prodigiosin, and to apply these findings to the following problems: (a) The syntrophic pigments produced by pairs of Serratia mutants, (b) Analogs of prodigiosin produced by mutant 9-3-3 on substituting other alkylmonopyrroles for 2-methyl-3-amylpyrrole, and (c) Prodigiosin-like compounds isolated from Serratia mutants and from other microorganisms.

REVIEW OF THE LITERATURE

Work on the characterization of prodigiosin was first reported by Wrede and Hettche (37) and Wrede (35). Four- to five-day cultures of Serratia marcescens were treated with 10 per cent NaOH and after the addition of alcohol the pigment was extracted with petroleum ether. After washing and filtering, the petroleum ether extract was concentrated to a small volume and dry hydrogen chloride gas was bubbled through to precipitate the pigment hydrochloride. The pigment was further purified by dissolving the hydrochloride in alcohol and adding 5 per cent $HClO_4$ to precipitate the perchlorate salt. The crystalline perchlorate had a melting point of 228°. Elementary analysis gave the empirical formula $C_{20}H_{25}N_3O \cdot HClO_4$. The pigment formed a stable zinc salt when $ZnSO_4$ was added to the culture medium. Spectroscopic and other properties of this salt were noted to be similar to the properties of dipyrrylmethene zinc salts. Zerewitinoff and Zeisel analysis by Wrede (36) indicated the presence of two active hydrogens and one methoxyl group, respectively.

In 1933 Wrede and Rothaas (38) reported the first degradative work done on prodigiosin. They were able to recover a pyrrole with the formula $C_{10}H_{17}N$ from a soda lime distillation of prodigiosin. Hydrogenation of the isolated compound gave a pyrrolidine, $C_{10}H_{21}N$. Oxidation of the pyrrole with CrO_3 in acetic acid (40) gave an oily product, n-amylmaleimide, which

after catalytic hydrogenation yielded a product shown to be identical to synthetic n-amylsuccinimide. Only one carbon was lost in the CrO_3 oxidation and it was thus concluded that the pyrrole contained a methyl group in the 2- position. Oxidation of the pyrrole with $KMnO_4$ in acetone gave only one acid, n-caproic acid. The pyrrole was therefore concluded to be a disubstituted pyrrole with a methyl group in the 2- position and an amyl group at either the 3- or the 4- position. Synthesis of 2-methyl-3-amylpyrrole and comparison to the cleavage pyrrole from the soda lime distillation showed the two to be identical, thus ruling out the 2,4 isomer.

Oxidation of prodigiosin with CrO_3 in acetic acid (39) yielded both maleimide and methoxymaleimide. Catalytic hydrogenation of prodigiosin followed by acid oxidation with $KMnO_4$ gave proline.

Wrede's degradative work indicated that there were three pyrrole components in prodigiosin: pyrrole, 2-methyl-3-amylpyrrole, and 3-methoxypyrrole. On the basis of his findings, he reasoned that there were two plausible structure types for the prodigiosin molecule, a tripyrrylmethene or a pyrryldipyrrylmethene. Wrede felt that the spectral analysis of prodigiosin was suggestive of a tripyrrylmethene structure and because of his assignment this structure was considered to be the correct one for a number of years.

In 1956 Santer and Vogel (23) reported the isolation of a pyrrole-containing precursor of prodigiosin. Serratia marcescens mutant 9-3-3 was found to produce a stable substance which permitted pigment formation in another colorless mutant, W-1. The compound gave a positive color reaction with the Ehrlich reagent, p-dimethylaminobenzaldehyde, indicating the presence of a pyrrole ring. The compound was also shown to contain a methoxyl and a carbonyl group. Elementary analysis was in good agreement with the empirical formula $C_{10}H_{10}N_2O_2$. That this pyrrole was precursor was demonstrated by tracer experiments. Strain 9-3-3 was grown in a medium supplemented with glycine-2-^{14}C. The pyrrole described above was isolated in crystalline form and found to be radioactive. It was supplied to strain W-1 and the red pigment formed was extracted and found to be labeled, its specific activity being equal to that of the labeled pyrrole supplied to strain W-1. Alkaline peroxide oxidation of the precursor by Wasserman et al. (27) yielded pyrrole-2-carboxamide, providing good evidence for a 2,2'-bipyrrole linkage. This evidence along with n.m.r. data suggested 4-methoxy-2,2'bipyrrole-5-carboxaldehyde as the most probable structure for the precursor. The acid-catalyzed condensation of the precursor with 2-methyl-3-amylpyrrole gave a product identical to prodigiosin and led Wasserman to propose a pyrryldipyrrylmethene type structure for prodigiosin.

In 1962, Rapoport and Holden (19) reported the complete synthesis of prodigiosin. This was accomplished by the synthesis of 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde followed by the acid-catalyzed condensation of this bipyrrole with 2-methyl-3-amylpyrrole. The synthetic product proved to be identical to the natural pigment. The pyrryldipyrrlmethene structure for prodigiosin was thus established.

Various physical properties of prodigiosin have been reported by a number of authors. As mentioned previously, Wrede and Hettche reported the melting points of several crystalline salts of prodigiosin, including the hydrochloride, perchlorate, and zinc salts. Hubbard and Rimington (11) were the first to publish ultraviolet-visible spectra of the acid and base forms of the molecule. They also reported approximate values for molar absorptivities at absorption maxima. Morgan and Tanner (16) confirmed with spectral values obtained by Hubbard and Rimington with several minor exceptions and also listed infrared absorption bands of both the free base and the perchlorate salt. Castro et al. (6) reported that the Morgan and Tanner infrared spectral data were incomplete, citing several absorption bands not reported in their paper. Castro also gave precise values for molar absorptivities of ultraviolet-visible maxima in 95 per cent ethanol acidified 95 per cent ethanol, isopropanol, and acidified isopropanol, noting an effect of solute concentration on the absorptivity in both 95 per cent

ethanol and isopropanol. A decrease in ϵ at 537 mu with dilution was accompanied by a rise in absorption in the neighborhood of the maximum for the free base, suggesting a proton exchange reaction involving the perchlorate and the alcohol, the alcohol functioning as a Lewis base. Burgus (5) has described the countercurrent distribution of the free base. In a solvent system consisting of petroleum ether:methylcellosolve:0.01 M phosphate buffer, pH 7.2 (4:3:1) a distribution coefficient of K = 0.82 was obtained.

Prodigiosin and prodigiosin-like compounds have also been reported in organisms other than Serratia marsescens. Dietzel (7, 8) isolated a prodigiosin-like compound from selected Streptomyces strains. Elementary analysis indicated an empirical formula of $C_{25}H_{35}N_3O$, an excess of 5 $-CH_2-$ groups when compared to the formula for prodigiosin, $C_{20}H_{25}N_3O$. The ultraviolet-visible absorption spectrum of the compound was similar to that of prodigiosin. From these data Dietzel surmised that his compound was probably a higher homolog of prodigiosin.

Wasserman (25) isolated a red pigment from Streptomyces longisporus ruber which was similar to the compound isolated by Dietzel. Cleavag with HI yielded a C_{15} pyrrole. The n.m.r. spectrum of the cleavage product indicated the presence of a $C-CH_3$ group. $KMnO_4$ oxidation gave C_4 and C_7 acids. Wasserman concluded that 2-methyl-3-heptyl-4-propylpyrrole was a plausible structure for the C_{15} pyrrole. That the pigment contained the same bipyrrole moiety as prodigiosin was shown

by synthesis of the C_{25} pigment from the condensation of the C_{10} aldehyde prodigiosin precursor with the C_{15} cleavage pyrrole. Infrared analysis showed the condensation product to be indistinguishable from the natural product. Wasserman later published a note (28) stating that he had synthesized authentic 2-methyl-3-heptyl-4-propylpyrrole and after condensation with the C_{10} bipyrrole precursor obtained a product differing from the natural pigment in both n.m.r. and infrared spectra. Thus it should be noted that the alkyl substitution on the monopyrrole moiety of this C_{25} pigment remains to be elucidated.

Another report of the isolation of a C_{25} prodigiosin-like pigment from certain Streptomyces strains came from Arcamone et al. (1). A countercurrent distribution system was described for parititioning the hydrochloride salt. In a solvent consisting of petroleum ether, benzene, ethyl alcohol, and 0.1 N HCl (4:1:3:2) a distribution coefficient of approximately unity was obtained. Ultraviolet-visible spectra were reported. The infrared spectrum was found to contain several marked differences from the spectrum of prodigiosin. Zeisel analysis indicated the presence of a methoxyl group (17). $KMnO_4$ oxidation in acetone yielded pyrrole-2-carboxylic acid and pyrrole-2,5-dicarboxylic acid, and CrO_3 oxidation of the pigment gave maleimide and methoxymaleimide, indicating the presence of two pyrrole muclei in the molecule. Elementary analysis agreed with that reported for the compounds isolated by Wasserman and

Dietzel. Indeed, it is probable that these workers were all dealing with the same compound. Certainly the data presented are consistent with this possibility. This viewpoint is supported by Wasserman (26) and Rapoport and Holden (19).

Recently, Khokhlova et al. (12) reported isolation of a C_{25} pigment from Actinomyces aureoverticillatus. On the basis of n.m.r. and ultraviolet-visible spectra, chromatographic behavior in a number of solvents, and elementary analysis they suggested that the pigment was either isomeric or identical to the C_{25} prodigiosin analog reported by other workers.

Another example of the occurrence of a prodigiosin-like compound was reported by Perry (18) who isolated from some Streptomyces strains a pigment which was chromatographically similar to prodigiosin on paper. The ultraviolet-visible spectrum was also similar to that of prodigiosin. These data led Perry to conclude that the pigment was prodigiosin. The aforementioned similarities in the chromatographic behavior and ultraviolet-visible spectrum of prodigiosin to the C_{25} prodigiosin analog indicate that Perry did not have sufficient data to distinguish between the two pigments. Considering the reported occurrence of the C_{25} pigment in various Streptomyces strains, it is probable that he had isolated the analog rather than prodigiosin.

Recently, evidence for the occurrence of prodigiosin itself in organisms other than Serratia marcescens was supplied

by Lewis and Corpe (14). They isolated gram-negative red-pigmented rod-shaped bacteria from a marine sediment sample. Morphological and physiological features of the organisms indicated that they were not members of the genus Serratia. Purified pigment from one of these marine isolates had ultraviolet-visible and infrared spectra identical to those of prodigiosin. Chromatographic behavior of the pigment on thin layer and paper was also similar to that of prodigiosin.

It should be noted that pigment extracts from wild-type Serratia marcescens itself are generally found to be heterogeneous on chromatography, prodigiosin being the major component (6, 33). It is possible that the minor components are prodigiosin-like compounds, but these have not yet been characterized. However, a compound differing from prodigiosin only in the replacement of the methoxyl group by a hydroxyl group, designated norprodigiosin, has been isolated as the major component of orange Serratia marcescens strain OF by Hearn et al. (10). The hydroxyl analog was much less stable than prodigiosin but could be converted to prodigiosin by treatment with diazomethane.

Finally, the only other prodigiosin-like compounds reported in the literature were two synthetically prepared isomers of prodigiosin made by Rapoport and Holden (19) during their work on the synthesis of prodigiosin. One of the isomers was prepared by the condensation of 3-methoxy-2,2'-bipyrrole-5-carboxaldehyde with 2-methyl-3-amylpyrrole. The product, with

methoxyl group in the three position rather than in the four position as in prodigiosin, had an ultraviolet-visible spectrum markedly different from the spectrum of prodigiosin, the isomer showing a 50 mμ shift in the absorption maximum from that of prodigiosin in acid solution, and a 10 mμ shift in basic solution. The other synthetic isomer was prepared by the condensation of 4-methoxy-2,2'bipyrrole-5-carboxaldehyde with 2-butyl-3-ethylpyrrole. This monopyrrole was obtained as a by-product from the synthesis of 2-methyl-3-amylpyrrole, from which it was readily separated by vapor phase chromatography. The isomer obtained from 2-butyl-3-ethylpyrrole had visible spectra very similar to prodigiosin; however, Rapoport did note a small shift of the main peak in the free base form and the absence of a low-extinction peak at 532 mμ which is present in the spectrum of prodigiosin.

Early studies of color variants of Serratia marcescens were reviewed by Bunting (4). Labrum and Bunting (13) found that the red HY strain of Serratia marcescens produced stable pink and white types as spontaneous mutants, but that the variants were found in larger numbers following exposure of parent cells to ultraviolet irradiation. Data on incidence, stability, and behavior suggested that the variants were true gene mutations. In a later presentation (3), Bunting further classified the prodigiosin-deficient HY color mutants as belonging to one of several classes, depending upon their ability to accumulate one or the other of the immediate prodigiosin precursors and

their ability to enzymatically couple the precursors to form prodigiosin.

Rizki (20) and Williams and Green (31) reported that some of the stable prodigiosin-deficient mutants of Serratia marcescens produced by ultraviolet irradiation could accumulate diffusible factors permitting red pigmentation to take place in certain different mutants of the same species otherwise unable to produce pigment. . .This phenomenon was termed chromogenic induction by Rizki and syntrophic pigmentation or cross-feeding by Williams and Green and by Santer and Vogel (23).

Identification of the syntrophic pigment produced by mutant pairs exhibiting cross-feeding was first attempted by Rizki (21), who after analysis of the ultraviolet-visible spectra of syntrophic pigment extracted from several mixed cultures reported that these spectra were similar to that of prodigiosin. Williams (32) found that syntrophic pigment from one pair of mutants behaved identically to authentic prodigiosin on paper chromatography. Santer and Vogel, as cited previously, reported labeled syntrophic pigment from the mutant pair 9-3-3 and W-1 to be prodigiosin on the basis of chromatographic behavior on paper and the visible absorption spectrum. Burgus (5), studying a pair of mutants in the bipyrrole pathway, namely, WCF and OF, concluded that the pigment produced by this pair was identical to prodigiosin on the basis of elementary analysis, countercurrent distribution, column chromatography, and comparison of infrared spectra.

Cross-feeding has been a valuable tool for studying prodigiosin biosynthesis; indeed, the only known precursors of the pigment have been discovered in this way. Thus Santer and Vogel isolated the precursor later identified by Wasserman as 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde. More recently, Hearn et al. (10) have isolated an unstable precursor from cultures of the orange mutant OF and subsequently identified it as 4-hydroxy-2,2'-bipyrrole-5-carboxaldehyde. It was concluded that this compound is the immediate precursor to Santer and Vogel's compound, to which it is converted by enzymatic methylation.

EXPERIMENTAL

Materials

Organisms

All mutants used in this work were prepared by ultraviolet irradiation of wild-type strains of Serratia marcescens. A number of wild-type strains of Serratia marcescens are known, all of which produce prodigiosin. Mutants WCF, OF, and H-462 were supplied by Dr. Robert P. Williams of Baylor University College of Medicine. From parent strain Nima were obtained a number of stable mutants including the non-pigmented white strain WCF and the orange variant OF, both of which have been described by Williams and Green (30). Mutant H-462, obtained from parent strain HY, is a non-pigmented mutant not described in the literature. It was chosen for this study because of (a) its ability to act as a receptor and form red pigment when grown with strain OF, and (b) its ability to act as a donor and stimulate red pigmentation in mutant WCF when the two are grown together.

Mutant 9-3-3, another colorless mutant also obtained by ultraviolet irradiation of parent strain HY, accumulates the final bipyrrole precursor in the biosynthesis of prodigiosin. This mutant was originally supplied by Dr. Mary I. Bunting of Radcliffe College and was obtained by us through Dr. Williams.

Media

Williams' medium minus phosphate salts The complete medium of Williams (31) minus phosphate salts consisted of: yeast extract, 0.1 per cent; enzymatic casein hydrolyzate, 0.2 per cent; glycerol, 1.0 per cent; ammonium citrate, 0.5 per cent; NaCl, 0.5 per cent; $MgSO_4 \cdot 7H_2O$, 0.05 per cent; and ferric ammonium citrate, 0.005 per cent; made up in deionized water. The final pH of the medium was adjusted to 7.0.

Harned's medium Harned's medium (9) consisted of: D-mannitol, 2.0 per cent; Bacto-Neopeptone, 1.0 per cent; and $MgSO_4 \cdot 7H_2O$, 0.125 per cent; made up in 40 per cent tap, 60 per cent deionized water (These proportions of tap and deionized water had been previously used because of the high mineral content in Iowa State University tap water). The final pH of the medium was adjusted to 5.0 with 3 N HCl.

Peptone-glycerol medium Peptone-glycerol medium (13) consisted of: Bacto-Neopeptone, 0.5 per cent; and glycerol, 1.0 per cent. The final pH of the medium was adjusted to 7.0.

Instruments and reagents

All reagents used were either CP or Analytical grade unless otherwise stated. Skelly B and methylcellosolve used for column chromatography and countercurrent distribution were routinely redistilled. The diatomaceous earth used for column chromatography was prepared according to the method of Worthington (34) from "Hy Flo Super-cel" (Johns-Mansville).

The 2,4-dimethylpyrrole and 2,4-dimethyl-3-ethylpyrrole (kryptopyrrole) were obtained from K & K Laboratories, Inc., 121 Express Street, Plainview, New York. The kryptopyrrole was redistilled under vacuum before using. The 2,4-dimethylpyrrole was not redistilled, since the n.m.r. spectrum of this compound as obtained from K & K indicated sufficient purity.

Carbon, hydrogen, and nitrogen analyses were carried out by Ilse Beetz Mikroanalytisches Laboratorium, 8640 Kronach, Postfach 460, West Germany.

Ultraviolet-visible spectra were obtained on either a Cary Model 14 or Perkin-Elmer Model 21 double beam infrared spectrophotometer. The n.m.r. spectra were determined with a Varian Model A-60 high resolution n.m.r. spectrometer.

Vapor phase chromatography was conducted on an Aerograph Model A-90-P2 gas chromatograph. The partitioning liquid phase was Silicone fluid (methyl) SF-96 on diatomaceous earth.

The countercurrent distribution apparatus used in this work was modeled after the micro-distribution machine described by Bell et al. (2). The machine consists of one row of 60 tubes, each with a 1-ml. upper phase, 1-ml. lower phase capacity. It was constructed in the glass shop of Iowa State University.

All pH measurements were conducted on a Beckman Model 76 expanded scale pH meter. Melting points were determined on a

MEL-TEMP capillary melting point apparatus. Samples used for determination of molar absorptivities were weighed on an Oertling Model 146 microanalytical balance.

Bacterial cells were harvested from broth cultures on either a Servall Model RC-2 or an International Model PR-2 refrigerated centrifuge.

Absorbance readings at a given wavelength in the visible range in countercurrent distribution runs were made on either a Bausch and Lomb Spectronic 20 colorimeter or a Beckman DU spectrophotometer.

General Methods

Cultures

Frozen stock and working cultures Initially, 3 ml. of Williams' medium was autoclaved in each of a number of screw-cap 15-ml. test tubes. The tubes were then carefully inoculated from Williams' agar stock slants of the individual variants and the cultures allowed to grow for 24 hours. They were then quick-frozen in a dry ice-acetone bath and stored in a freezer. Working culture tubes were perpetuated by inoculating additional 3-ml. Williams' medium tubes from thawed culture tubes with a needle, allowing these cultures to grow for 24 hours, and quick-freezing.

Broth cultures Sterile, cotton-stoppered 2-1. Erlenmeyer flasks of Harned's, Williams' minus phosphate, or peptone-glycerol medium were inoculated with 2-5 ml. of a 24-hour tube inoculum of the respective medium. The 24-hour tube inoculum had been inoculated by thawing a 3-ml. frozen working culture tube and inoculating a screw-cap test tube containing 10 ml. of the desired medium with a needle or by aseptically pipetting 0.1 ml. of the 24-hour working culture into the screw-cap tube. The flasks were shaken for the desired length of time in a New Brunswick Model G25 incubator shaker at 28°C. at a speed setting of 6.

Extraction of prodigiosin and prodigiosin-like compounds; hydrochloride salt formation

Prodigiosin and prodigiosin-like pigments were isolated from broth cultures by a modification of the basic saponification method of Wrede (35). Cells were harvested by centrifugation. When the Servall centrifuge was used the liquid cultures were centrifuged at 7,000 r.p.m. for 25 minutes. Using the International centrifuge, cells were collected by centrifugation at 4,000 r.p.m. for 35 minutes. The harvested cells were washed with distilled water into a beaker. This cellular mud was treated with an equal volume of 10 per cent NaOH and stirred for 2 hours. A volume of 95 per cent ethanol equal to the total volume of the mixture was then added and stirred continually for an additional 30 minutes. This mixture

was repeatedly extracted with half volumes of Skelly B in a separatory funnel. The combined Skelly B extracts were concentrated to approximately 40-50 ml. on a roto-evaporator. Anhydrous Na_2SO_4 was added and after shaking the mixture was allowed to stand for several hours. The Na_2SO_4 was filtered off and dry hydrogen chloride gas was bubbled through the dried Skelly B-pigment solution depositing the pigment hydrochloride as a red amorphous precipitate in nearly quantitative yield. The hydrochloride was collected by centrifugation and dried in a vacuum desiccator. Further purification of pigments is discussed in later sections.

Countercurrent distribution

Countercurrent distribution experiments were carried out on the free base forms of the pigments. One ml. of lower phase of the 2-phase solvent system was added to all tubes of the countercurrent distribution machine except for the first tube. To this tube was added the sample dissolved in 1 ml. of lower phase. One ml. of upper phase was then added to this first tube. The tubes were agitated, completely mixing the phases. The shaking was stopped and the phases allowed to separate. The upper phase was then transferred to the next tube, and the first tube was then recharged with another 1-ml. portion of upper phase. This process was repeated until the desired number of transfers were effected. After the run was completed, the combined upper and lower phases were collected from each

tube. A standard amount of acetone was added to each tube to insure formation of a single phase. A drop of 3 N HCl was added to each tube and the absorbance was determined at the acidic absorption maximum.

To find the approximate partition coefficient K from the experimental plots, the following expression was used: $K = \frac{N}{n - N}$, where N is the number of the peak tube (not necessarily integral), and n is the total number of transfers of the run.

Thin layer chromatography

Thin layer chromatography on microscope slides employing Silica Gel G as the adsorbent was routinely used in the characterization of pigments and degradative products obtained from the pigments. Glass miscroscope slides (3 x 1 inch) were placed in a shallow plexiglas aligning tray made by the Iowa State Chemistry Shop. A slurry was made by shaking a mixture of Silica Gel G and distilled water (2:1). Application of this slurry was done with an aluminum spreader block, also made by the Iowa State Chemistry Shop. The thin layer slides were allowed to air dry for a few minutes and then heated at 110° for 1 hour to activate the thin layer. Samples were applied with micropipettes made from drawn out capillary tubing. The plates were developed in small solvent-saturated glass developing tanks.

Detection of pyrroles

Pyrroles were detected by the Ehrlich reaction. In this reaction, a colored compound is formed by acid-catalyzed condensation of p-dimethylaminobenzaldehyde with the pyrrole. Pyrroles with free alpha-positions react with the aldehyde to give blue to red colored dyes. For spraying thin layer chromatograms, a 1 per cent methanolic solution of p-dimethylaminobenzaldehyde was diluted 1 to 5 with 1 N methanolic HCl (86 ml. of concentrated HCl diluted to 1 l. with absolute methanol).

Syntrophic Pigments

General observations

One of the purposes of this investigation was to verify the sequence of steps blocked in mutants lacking the complete bipyrrole pathway necessary for production of prodigiosin. To do this it seemed necessary to show that the syntrophic pigment produced by mutant pairs was identical to the wild-type pigment, i.e., prodigiosin. Mutants WCF, H-462, and OF were chosen for study. The pattern of syntrophic pigment production exhibited by these mutants was confirmed in this laboratory by Bascur de Medina (15). As stated in the Introduction, mutant OF was shown to be a donor to both WCF and H-462, H-462 was shown to be a receptor to OF and a donor to WCF, while WCF was scored as a receptor to both of the other mutants. The syntrophic pigment produced by mutants OF and WCF when grown

together on surface cultures was shown by Burgus to be identical in a number of ways to prodigiosin. In the present study, the syntrophic pigments produced by the mutant pairs WCF/H-462 and OF/H-462 were isolated and their properties compared to those of prodigiosin.

Production and isolation of syntrophic pigments

Production of syntrophic pigments were accomplished through the growth of mixed-mutant liquid broth cultures. For both mutant pairs the following procedures were followed: In a typical experiment, 12 2-l. Erlenmeyer flasks containing 400 ml. of Harned's broth were autoclaved. Half of the flasks were then inoculated with a 5-ml. 24-hour Harned's culture of one of the mutants of the pair being studied, which had in turn been inoculated with a needle from a 24-hour working culture. The remaining 6 flasks were inoculated in the same way with a comparable culture of the other mutant. The flasks were placed in a New Brunswick Model G25 incubator shaker and shaken for 24 hours at 28° at a speed setting of 6. After 24 hours, growth was visible in all flasks. In the case of mutant OF, the 24-hour liquid culture was orange. WCF and H-462 24-hour cultures were colorless. The cultures were then mixed by aseptically transferring the 24-hour culture of one of the mutants to the liquid culture of the other mutant. Red pigmentation was immediately visible in the 6 flasks now each containing 800 ml. of mixed culture. The flasks were shaken

for another 24 hours. At the end of this time pigmentation was observed to be very heavy. The cells were harvested by centrifugation and the pigment extracted into Skelly B according to the basic extraction procedure of Wrede. The pigment hydrochloride was formed from the Na_2SO_4-dried, concentrated Skelly B extract as described in the General Methods section.

Purification of syntrophic pigments

The pigment hydrochlorides were converted to the perchlorate salts in both cases in the following manner: The hydrochloride was dissolved in 95 per cent ethanol. The solution was heated and filtered to remove any insoluble impurities. Five per cent $HClO_4$ was added dropwise with stirring to the hot solution. The perchlorate salt crystallized as small purple needles which were birefringent when viewed through a polarizing microscope. After standing for 1-2 hours in an ice bath, the product was collected by suction filtration on a small Buchner funnel, washed several times with cold distilled water, dried, and finally washed several times with Skelly B. The product was recrystallized twice from hot 95 per cent ethanol and 5 per cent $HClO_4$. From a typical 12-flask experiment the yield of twice-recrystallized perchlorate salt was 120-150 mg. The dry weight of lyophilized cells from such an experiment averaged 21-25 g.

The pigments were further purified by column chromatography on diatomaceous earth. A 2 cm. x 12 cm. column was packed from slurry with Skelly B and Super-cel. Packing was aided by gentle tamping and by air pressure applied to the top of the column. The pigment to be chromatographed was first converted to the free base in the following way: About 50 mg. of pigment perchlorate was dissolved in 5 ml. of hot 95 per cent ethanol. One N NaOH was added dropwise until the solution turned brown. Two ml. of water was added and the solution extracted 4 times in a separatory funnel with 3-4 ml. portions of Skelly B. The combined Skelly B extracts were dried over anhydrous Na_2SO_4 for several hours, then concentrated to dryness on a roto-evaporator. The pigment was redissolved in 2-3 ml. of Skelly B and applied to the top of the column. Development of the column with Skelly B separated an orange fraction which trailed into a red fraction. The trailing red band arose as a result of partial conversion of the free base of the pigment to the acid form as it passed through the diatomaceous earth column. The orange band, which appeared to be the major component, was eluted with Skelly B. Elution of the red band required 0.25 per cent methanol in Skelly B. The collected orange and red fractions were combined and evaporated to dryness on a roto-evaporator. The pigment was redissolved in hot 95 per cent ethanol and the perchlorate salt was formed by dropwise addition of 5 per cent $HClO_4$. After cooling 1-2 hours the crystalline product was collected by

centrifugation, washed several times with water, and dried over $CaSO_4$ (Drierite) in a vacuum desiccator. From the original 50 mg. perchlorate sample was obtained about 30 mg. of column-purified perchlorate; i.e., yield from the column was about 60 per cent.

Comparison of syntrophic pigments to prodigiosin

Ultraviolet-visible spectra of wild-type prodigiosin, the OF/H-462 pigment, and the WCF/H-462 pigment are presented in Figures 1-3. The spectra were determined in both acidic ethanol (95 per cent ethanol 0.01 N in HCl) and basic ethanol (95 per cent ethanol 0.01 N in NaOH). The spectra of both syntrophic pigments were identical to those of prodigiosin in both acidic and basic solutions.

Molar absorptivities at the acidic absorption maximum of the syntrophic pigments (λ = 537 mμ) were obtained from the following expression: $\epsilon = \frac{A}{(c)(1)}$ where A = absorbance, c = concentration in moles per liter, and 1 = the distance in cm. that light travels through the solution. The values obtained are presented in Table 1.

The infrared spectrum of the perchlorate salt of the two syntrophic pigments was determined on KBr pellets. Spectra of the perchlorate salt of the syntrophic pigments and of prodigiosin are shown in Figure 4. In each case, the spectrum was identical to that of prodigiosin.

Figure 1. Ultraviolet-visible spectrum of prodigiosin

——— Spectrum in 95 per cent ethanol 0.01 N in HCl

– – – Spectrum in 95 per cent ethanol 0.01 N in NaOH

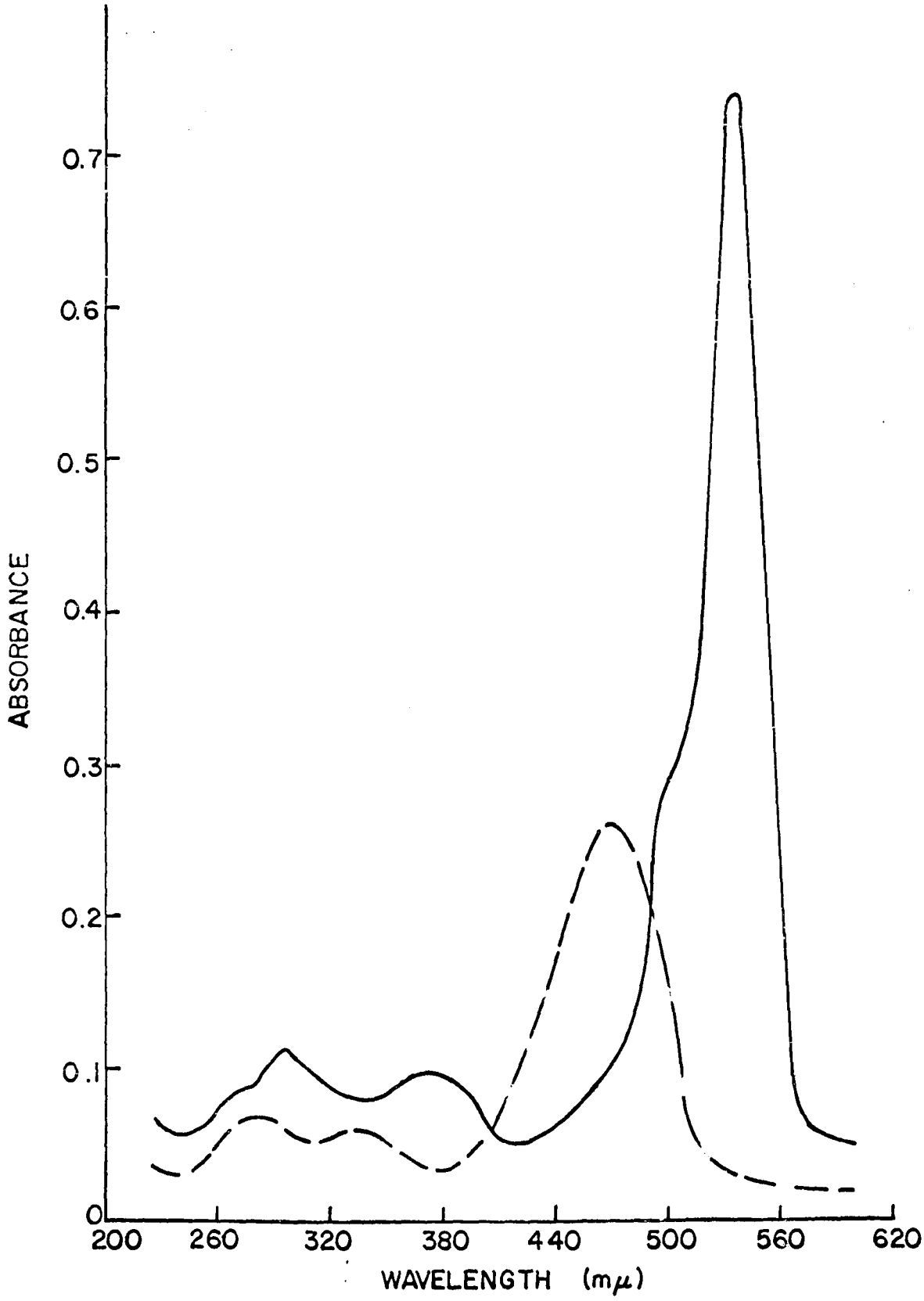

ABSORBANCE
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
200
260
320
380
440
500
560
620
WAVELENGTH (mμ)

Figure 2. Ultraviolet-visible spectrum of OF/H-462 pigment

——— Spectrum in 95 per cent ethanol 0.01 N in HCl

– – – Spectrum in 95 per cent ethanol 0.01 N in NaOH

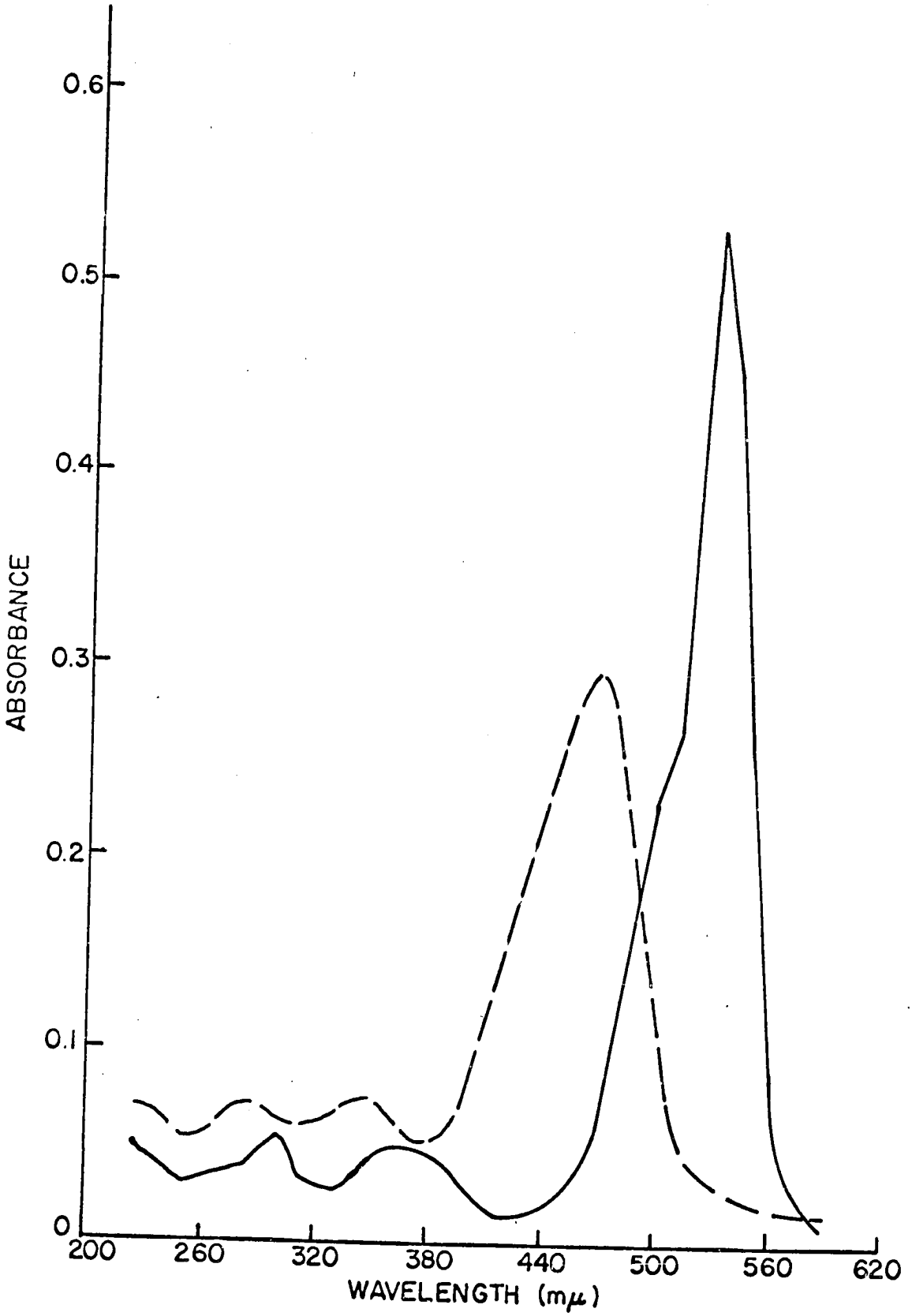

0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
200
260
320
380
440
500
560
620
WAVELENGTH (mμ)

Figure 3. Ultraviolet-visible spectrum of WCF/H-462 pigment

——— Spectrum in 95 per cent ethanol 0.01 N HCl

– – – Spectrum in 95 per cent ethanol 0.01 N NaOH

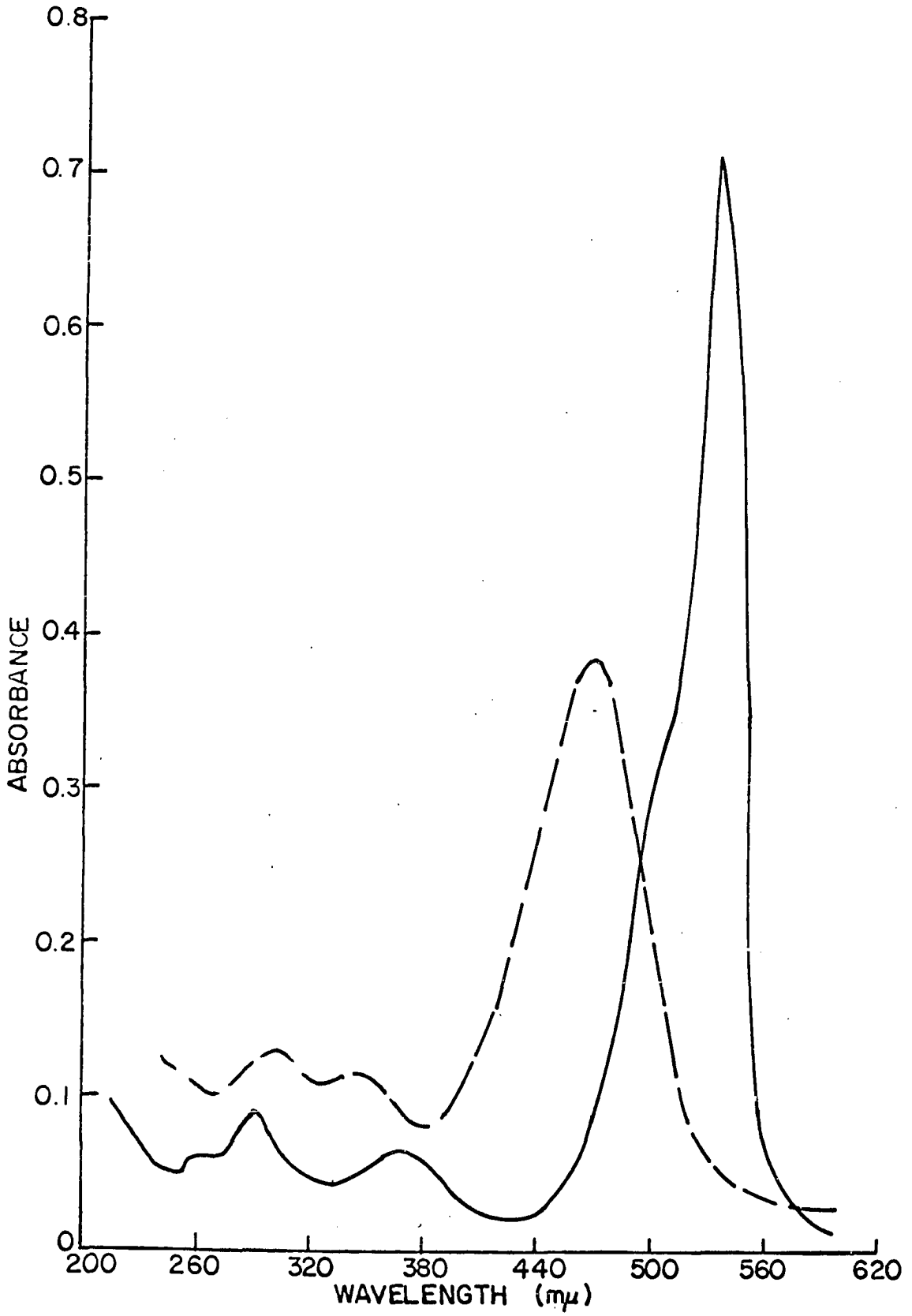

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
200
260
320
380
440
500
560
620
WAVELENGTH (mμ)

Table 1. Molar absorptivities of syntrophic pigments and prodigiosin

Sample	c(moles/l.)	$A_{537m\mu}$	Solvent	ϵ_{537}
Prodigiosin perchlorate[a]	———	——	acid ethanol	11.6×10^4
OF/H-462 pigt. perchlorate[b]	6.81×10^{-6}	0.74	acid ethanol	10.9×10^4
WCF/H-462 pigt. perchlorate	6.21×10^{-6}	0.67	acid ethanol	10.8×10^4

[a]Source: Castro et al. (6).

[b]The perchlorate salt used for this calculation was twice-recrystallized product which had not been subjected to column chromatography. Spectral analysis indicated this product to be sufficiently pure as such.

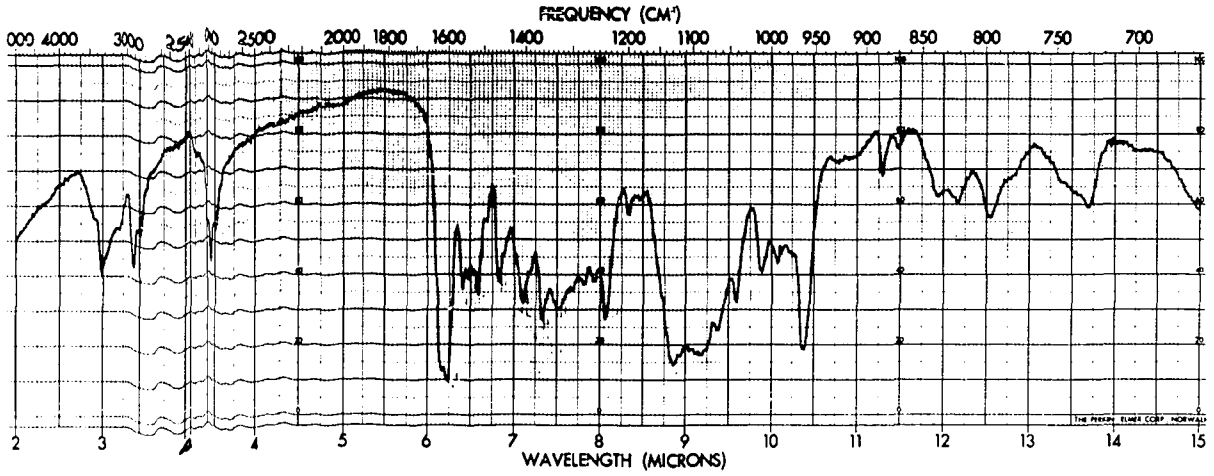
Twenty-transfer countercurrent distribution experiments in the solvent system described by Burgus (Skelly B, methylcellosolve, and 0.01 M phosphate buffer, pH 7.2; 4:3:1) afforded the distribution curves shown in Figures 5 and 6. In each case, the pigment perchlorate was dissolved in 1 ml. of lower phase solvent and 1 drop of 1 N NaOH was added to convert the pigment to the free base form. After the 20 transfer run was completed, the contents of the tubes were collected separately and 4 ml. of acetone and 1 drop of 3 N HCl was added to each tube. The absorbance of each tube was read at 537 mμ, the visible acidic absorption maximum for both

Figure 4. Infrared spectra in KBr

Top. Prodigiosin perchlorate

Center. OF/H-462 pigment perchlorate

Bottom. WCF/H-462 pigment perchlorate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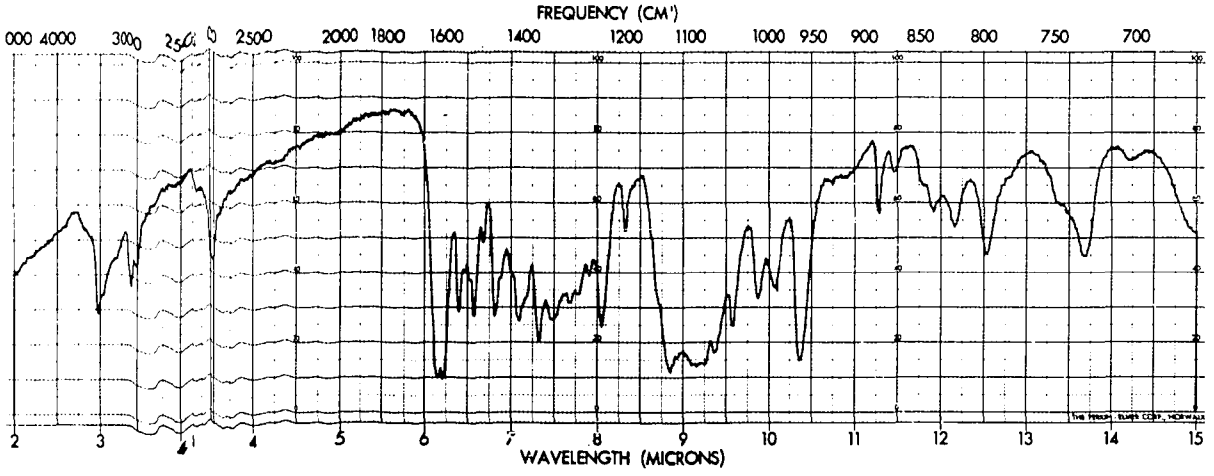
WAVELENGTH (MICRONS)


FREQUENCY (CM⁻¹)
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2 3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

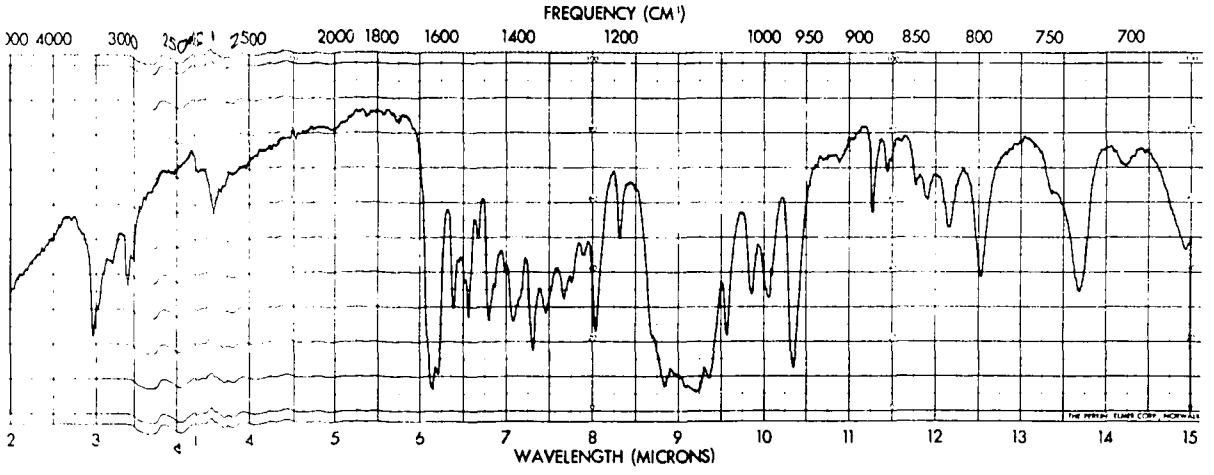

FREQUENCY (CM⁻¹)
4000 3000 2500 2000 1800 1600 1400 1200 1000 950 900 850 800 750 700
2 3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

Figure 5. Countercurrent distribution curve of OF/H-462 pigment in Skelly B, methylcellosolve, 0.01 M phosphate buffer, pH 7.2 (4:3:1)

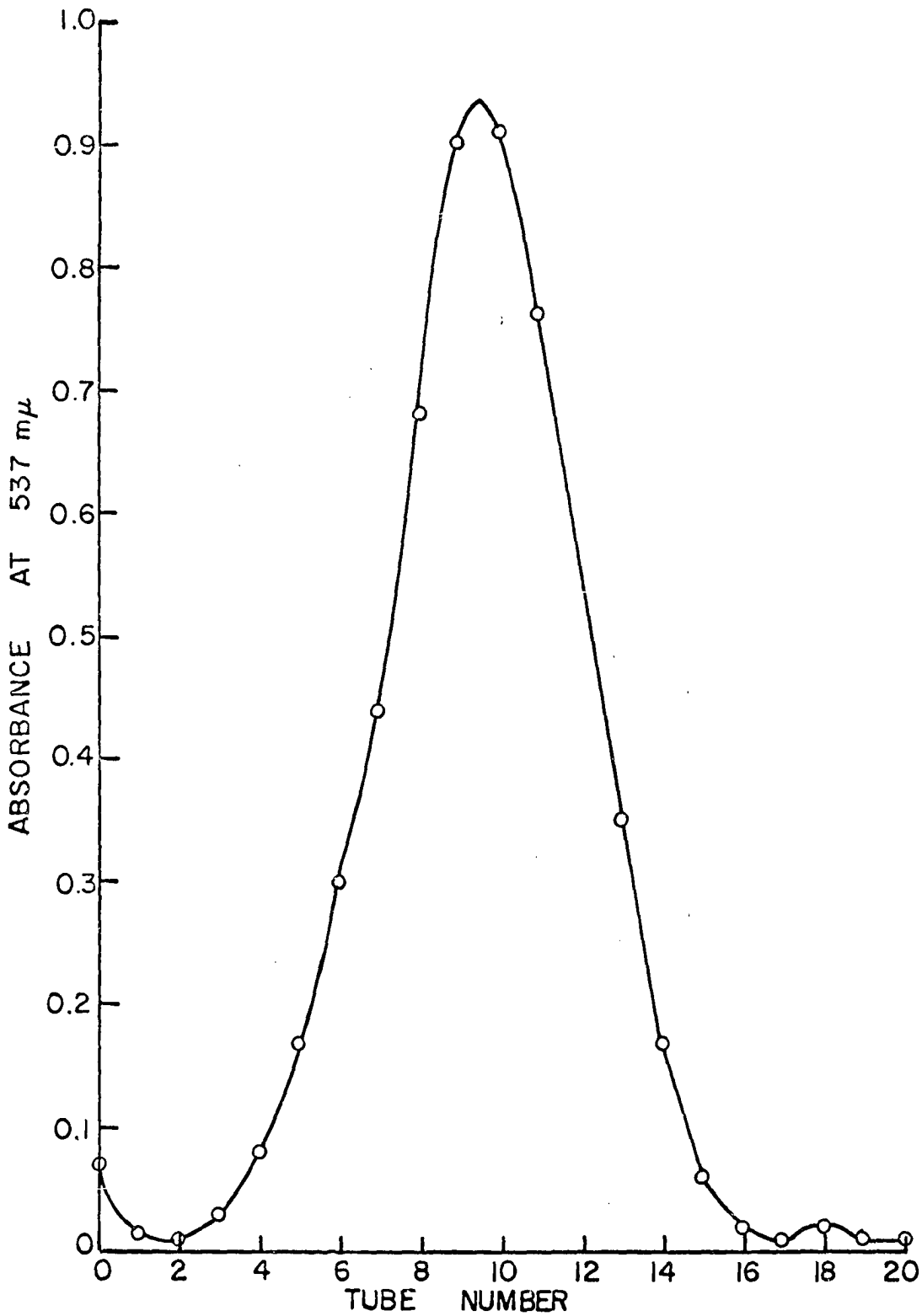

ABSORBANCE AT 537 mμ
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
16
18
20
TUBE NUMBER

Figure 6. Countercurrent distribution curve of WCF/H-462 pigment in Skelly B, methylcellosolve, 0.01 M phosphate buffer, pH 7.2 (4:3:1)

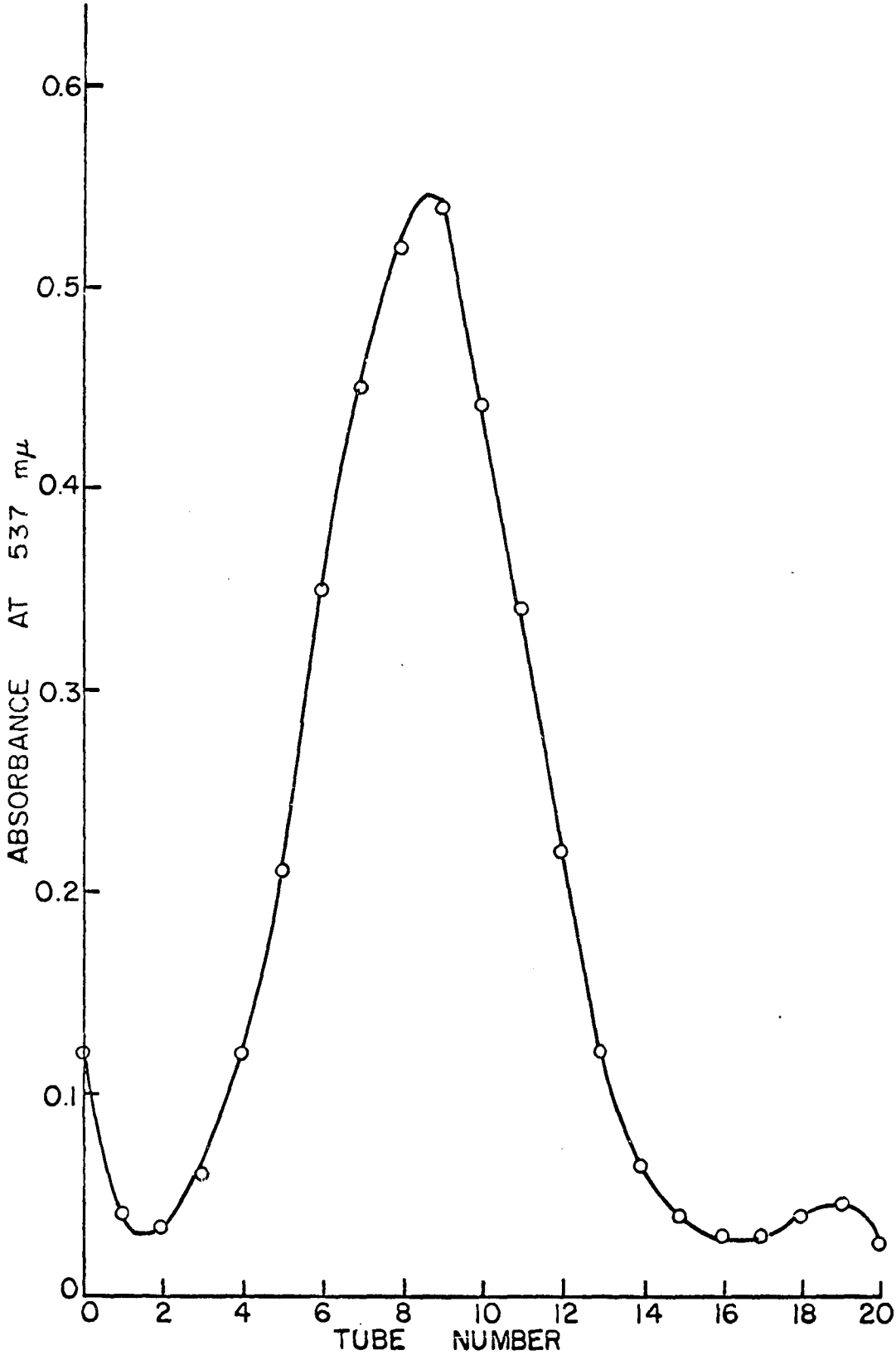

ABSORBANCE AT 537 mμ
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
16
18
20
TUBE NUMBER

Table 2. Elemental analysis of syntrophic pigment

Element	Calculated ($C_{20}H_{25}N_3O \cdot HClO_4$)	Found (OF/H-462 pigt.)	Found (WCF/H-462 pigt.)
Carbon	56.60	57.35	57.17
Hydrogen	6.13	6.17	6.31
Nitrogen	9.91	9.73	9.75

pigments. Both pigments had K values similar to that obtained for prodigiosin. The OF/H-462 pigment had a value of K = 0.9, while the WCF/H-462 pigment had a value of K = 0.8. Burgus reported a value of K = 0.8 for prodigiosin in this solvent system (5).

The syntrophic pigments were compared to prodigiosin by thin layer chromatography. Both pigments migrated identically to prodigiosin in ethyl ether, ethyl acetate, and methanol.

Carbon, hydrogen, and nitrogen analyses of the syntrophic pigment perchlorate salts were obtained. Results are presented in Table 2.

Soda lime distillation of syntrophic pigments and prodigiosin

Wrede reported (36) the recovery of 2-methyl-3-amylpyrrole from the soda lime distillation of prodigiosin. In a modification of his procedure, samples of the free base of both

syntrophic pigments and of prodigiosin were subjected to soda lime distillation and the cleavage products compared by vapor phase and thin layer chromatography.

The following procedure was used for each sample: 30 mg. of pigment free base was finely ground with 120 mg. of soda lime. A thick-walled 1 cm. x 15 cm. capillary tube was drawn out on one end after heating in an oxygen flame. The drawn out end was bent at 90° to the rest of the tube. The tube was loaded in the following manner: A silver wool plug was pushed as far into the tube as possible i.e., to the point where the tube was drawn out. This was followed by a 3-cm. layer of coarsely ground soda lime, then the soda lime-pigment finely ground mixture (also approximately 3 cm.) and finally another 3-cm. layer of coarsely ground soda lime. The charged tube was positioned in a simple oven constructed from a perforated stainless steel box covered with aluminum foil. A heating lamp was placed inside the oven and a thermometer placed in a position the same distance from the lamp as the sample tube. The drawn out end of the tube was submerged in a small test tube of ether which was in turn cooled in an ice bath. A stream of hydrogen was passed through the tube and the temperature gradually raised to 250°. Colorless liquid droplets were seen distilling over into the ether solution. The temperature was kept at 250° for 5 minutes and the apparatus was then allowed to cool. Some product had condensed beyond the silver plug which was not swept into the ether by the hydrogen stream.

This was combined with the rest of the distillate by breaking the tube at a point just beyond the plug and washing the condensate into the ether-distillate solution with additional ether. The ether solution was dried over anhydrous Na_2SO_4 and subjected to vapor phase chromatography.

Vapor phase chromatography of 10 µl. samples of the ether-distillate solutions from both syntrophic pigments and from prodigiosin afforded separation of one major component from all three samples which had very similar retention times and which was presumably 2-methyl-3-amylpyrrole. Several minor components were also detected. Vapor phase chromatography data are presented in Table 3. The absence of the short retention time minor component (0.82-0.87 min.) in the OF/H-462 pigment distillate-ether sample can be explained by the fact that the peak caused by this component has a retention time very close to that of the large ether peak, and in this run was probably obscured by it. It should also be noted that the peaks attributed to the other minor component were badly skewed, perhaps because the samples were not sufficiently dry at the time they were run; the presence of water can skew a peak because of steam distillation on the column packing. The major peak was well defined and nearly identical in all three samples.

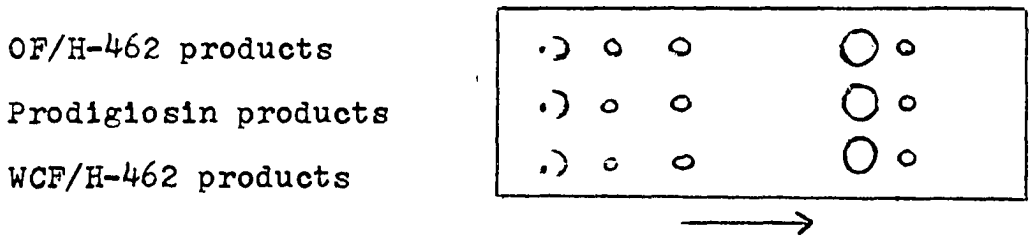
Thin layer chromatography on Silica gel G microscope slides in benzene indicated that the cleavage products were identical from all three samples. The distillate-ether solutions were

Table 3. Vapor phase chromatography of soda lime distillation from syntrophic pigments and prodigiosin

Sample	Column temp.	Detector temp.	Injector temp.	Flow rate	Retention times (average of 2 runs)
Prodigiosin distillate	158°	219°	220°	110 ml. per min.	0.82 min. (minor) 1.55 min. (minor) 5.75 min. (major)
OF/H-462 pigment distillate	158°	219°	220°	110 ml. per min.	1.58 min. (minor) 5.78 min. (major)
WCF/H-462 pigment distillate	158°	219°	220°	110 ml. per min.	0.87 min. (minor) 1.35 min. (minor) 5.83 min. (major)

spotted and the plates developed in benzene. The developed chromatogram was dried and sprayed with Ehrlich reagent, revealing the presence of one major red Ehrlich-positive component with Rf identical to that of synthetic 2-methyl-3-amylpyrrole. The Rf values of a number of alkylmonopyrroles available in this laboratory are so similar in a variety of solvents that thin layer chromatography alone does not provide positive identification; however, the thin layer results confirmed the vapor phase chromatography results with both syntrophic pigments. In addition to the major component, 4

other Ehrlich-positive spots were observed, although Wrede did not mention detecting any cleavage products from soda lime distillation of prodigiosin other than 2-methyl-3-amylpyrrole. The following drawing illustrates the Ehrlich-sprayed chromatogram of all three samples run in benzene:



green (Rf = 0.05) red (Rf = 0.68)
yellow (Rf = 0.15) pink (Rf = 0.80)
gray (Rf = 0.27)

It has been observed in this laboratory that 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde does not move from the origin on thin layer chromatography in benzene but yields a green spot when sprayed with Ehrlich reagent. The green, yellow, and gray spots all have Rf values less than 0.3; it is possible that these three spots represent this bipyrrole and/or degradative products of it. The low yield of cleavage products unfortunately makes characterization of minor components very difficult.

Pigment Production in a *Serratia marcescens* Mutant

General observations

The normally white mutant, strain 9-3-3, accumulates the immediate bipyrrole precursor to prodigiosin, 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde. Inorganic phosphate contained in the complete medium of Williams' has been shown to inhibit production of this compound when the mutant is grown in broth cultures. Omission of the phosphate salts from the medium removed the inhibition but also caused formation of a purple pigment (29).

In the present study the purple pigment was isolated and separated into several components. One of these components was shown to be identical to prodigiosin.

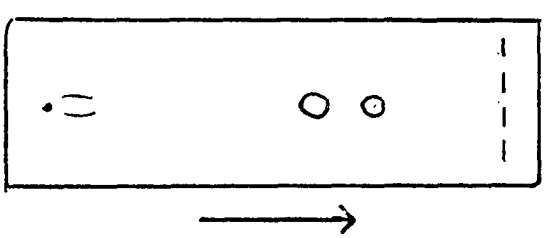
Production of purple pigment

Production of purple pigment was accomplished through the growth of strain 9-3-3 liquid Williams' minus-phosphate broth cultures. In a typical experiment the following procedure was followed: Twelve 2-l. Erlenmeyer flasks each containing 200 ml. of Williams' minus-phosphate broth described in the Materials section were autoclaved. The flasks were inoculated with a 2-ml. 24-hour Williams' minus-phosphate culture of strain 9-3-3, which had in turn been inoculated with a needle from a 24-hour working culture. The flasks were shaken for 48 hours at 28° at a speed setting of 6 in a New Brunswick Model G25

incubator shaker. At the end of this time the liquid culture appeared deep-purple. The cells were harvested by centrifugation and the pigment extracted from the cellular mud by one or the other or 2 extraction procedure, to be described in the following sections.

Acetone-ethyl acetate extraction of purple pigment

Extraction of purple pigment from 9-3-3 cells with acetone and ethyl acetate was accomplished in the following way: To the cellular mud from a 48-hour 12-flask experiment was added a volume of acetone equal to 3 times the volume of mud. The mixture was stirred for 20 minutes. The lysed cell debris was centrifuged and discarded. The aqueous acetone-pigment solution was concentrated on a roto-evaporator to a small volume, and the resulting aqueous pigment suspension extracted with ethyl acetate, in which the pigment proved to be very soluble. The ethyl acetate-pigment solution was evaporated to dryness and the pigment stored in a vaccum desiccator. Thin layer chromatography of the crude pigment on Silica gel G in ethyl acetate afforded the separation of several components, as illustrated in the following drawing:



red (Rf = 0.69)
blue (Rf = 0.78)

When run in the same thin layer system, prodigiosin had an Rf of 0.72, similar to the red component in the chromatogram drawn.

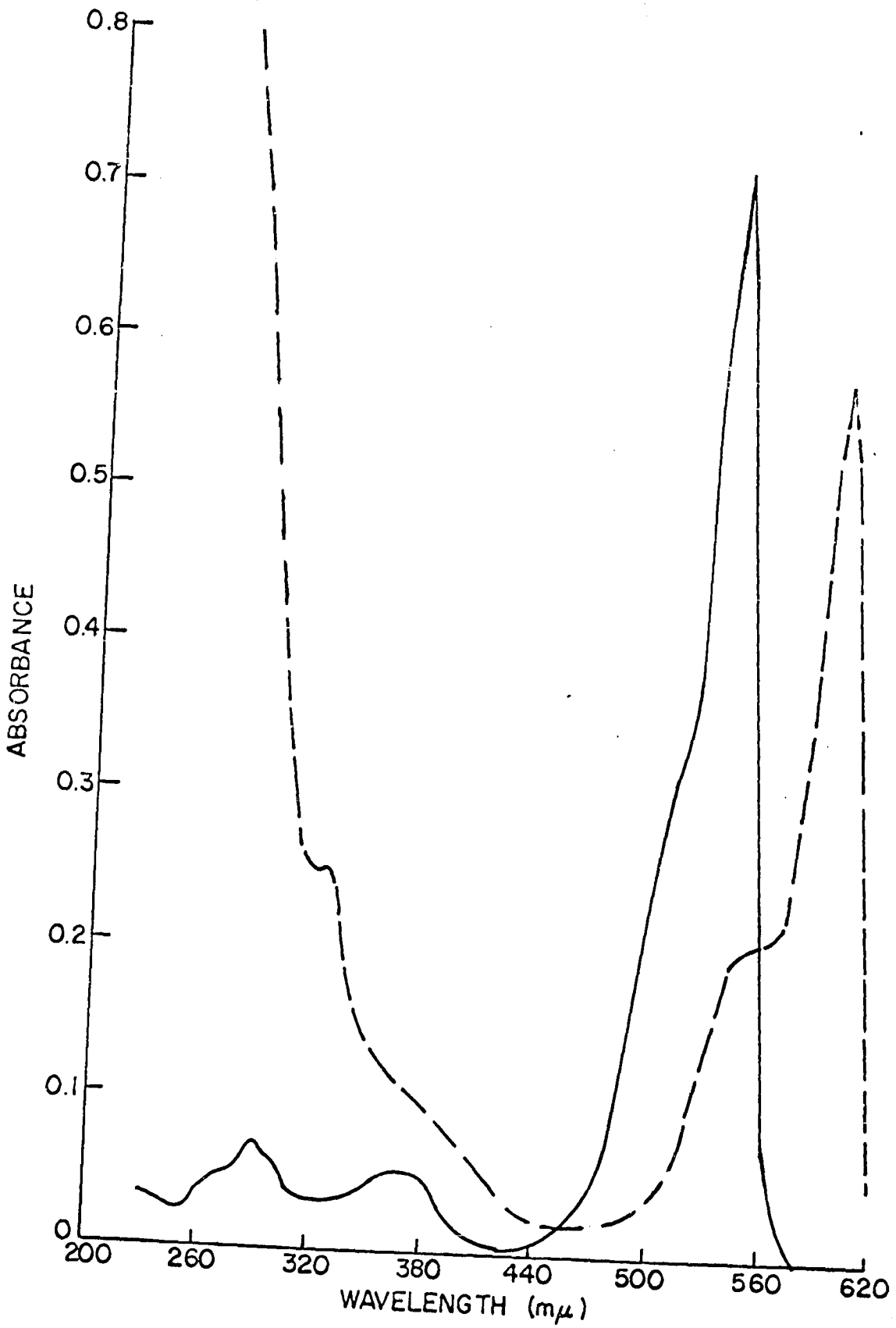
Column chromatography of crude purple pigment on silicic acid:celite (2:1)

A 2 cm. x 15 cm. column consisting of silicic acid:celite filter aid (2:1) was poured from slurry with chloroform. A 40-mg. sample of crude purple pigment from the acetone-ethyl acetate extraction was dissolved in 2-3 ml. of chloroform and applied to the top of the column. Development with chloroform yielded a fast-moving blue band followed by a red band. A large quantity of gray-black material remained at the top of the column. The blue and red fractions were collected separately. Thin layer chromatography of the red fraction on Silica gel G in methanol showed this component to behave identically to prodigiosin. Ultraviolet-visible spectra of both components are presented in Figure 7. Samples were prepared by evaporating the column fractions to dryness and redissolving them in acid ethanol. The blue component had an absorption maximum in the visible region of the spectrum at 595 mμ; the maximum observed for the red component was at 537 mμ with a shoulder at 505 mμ. It was noted that if the blue component was left in acid ethanol at room temperature for 72 hours, the absorbance at 595 mμ was markedly reduced, indicating the instability of this pigment in acid solution.

Figure 7. Ultraviolet-visible spectrum of red and blue pigment fractions obtained from fractionation of 9-3-3 purple pigment

——— Red fraction in 95 per cent ethanol 0.01 N in HCl

– – – Blue fraction in 95 per cent ethanol 0.01 N in HCl


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
200
260
320
380
440
500
560
620
WAVELENGTH (mμ)

Acetone-chloroform extraction of purple pigment and preparation of salts

Extraction of purple pigment from 9-3-3 cells with acetone and chloroform was accomplished in the following way: To the cellular mud from a 48-hour 12-flask experiment was added a volume of acetone equal to 3 times the volume of mud. After stirring for 20 minutes the lysed cell debris was centrifuged and discarded. To the aqueous acetone-pigment solution was added an equal volume of chloroform. The mixture was shaken gently and the phases allowed to separate, yielding a purple upper layer and a red lower chloroform layer. The lower layer was drawn off and dried over anhydrous Na_2SO_4. The Na_2SO_4 was filtered off and the solution evaporated to dryness on a roto-evaporator. The pigment was redissolved in about 50 ml. of 95 per cent ethanol and 1 N NaOH was added dropwise until the red solution turned yellow. Twenty-five ml. of distilled water was added and the solution extracted 4 times with 50-ml. portions of Skelly B. The pigment transferred readily from the basic ethanolic solution into Skelly B. The Skelly B extracts were pooled, concentrated to 20-30 ml. and dried over anhydrous Na_2SO_4. After filtering off the Na_2SO_4, dry hydrogen chloride gas was passed through the Skelly B-pigment solution. The hydrochloride clumped out as an amorphous red precipitate which was collected by centrifugation and dried in a vacuum desiccator; yield, approximately 60 mg.

The hydrochloride was dissolved in 10 ml. of 95 per cent ethanol, heated to near boiling, and filtered to get rid of any insoluble material. Hot 5 per cent $HClO_4$ was added dropwise until the solution became turbid. The turbid solution was cooled in an ice bath for 1 hour and the product collected by centrifugation. The product was washed with cold distilled water 3 times, dried in a vacuum desiccator, and recrystallized from 95 per cent ethanol and 5 per cent $HClO_4$; yield, 45 mg.

Comparison of 9-3-3 prodigiosin-like component to prodigiosin

The ultraviolet-visible spectrum in acid ethanol of the red component obtained from strain 9-3-3 is presented in Figure 7. The spectrum was identical to that of authentic prodigiosin isolated from wild-type strain Nima.

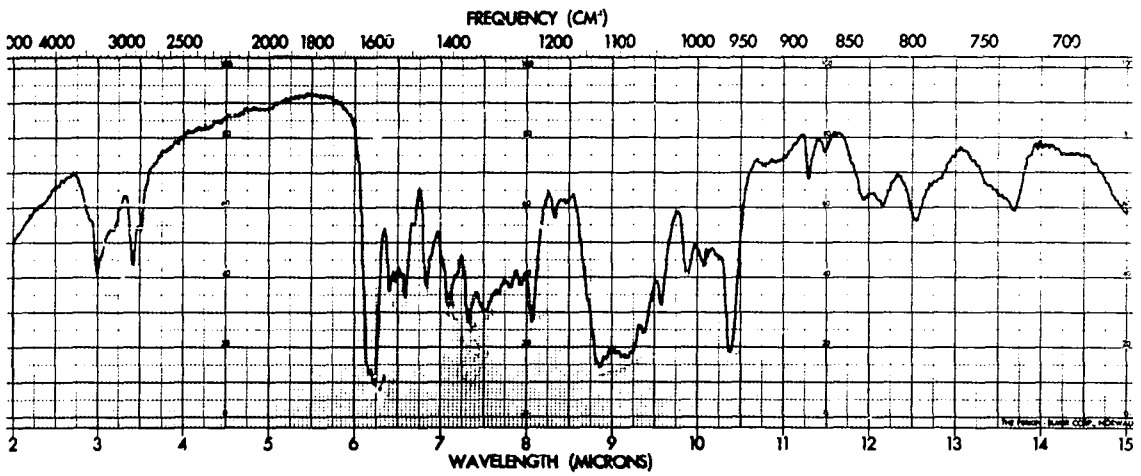
The molar absorptivity of the pigment perchlorate at the acidic visible absorption maximum 537 mμ was determined in acid ethanol. A solution containing 5.71×10^{-6} moles per liter gave an absorbance of 0.620 and was found to have a molar absorptivity of 10.9×10^{4}. This is close to the value of 11.6×10^{4} reported by Castro et al. and cited in Table 1 for prodigiosin.

The infrared spectrum of the perchlorate salt is presented along with the spectrum of the perchlorate salt of prodigiosin in Figure 8. The 2 spectra were observed to be identical.

Figure 8. Infrared spectra in KBr

Top. Prodigiosin perchlorate

Bottom. 9-3-3 prodigiosin-like pigment perchlorate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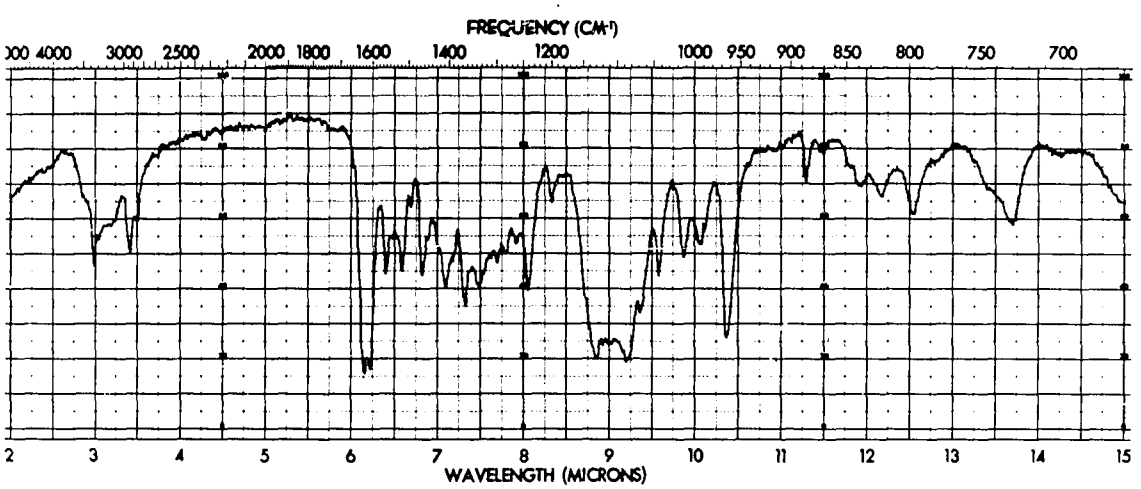
WAVELENGTH (MICRONS)


FREQUENCY (CM-1)
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2 3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

The pigment perchlorate was subjected to countercurrent distribution. A 20-transfer run in the solvent of Burgus described in the syntrophic pigment experimental section yielded a partition coefficient K of 0.7, close to that obtained by Burgus for authentic prodigiosin in the same system. A typical countercurrent distribution curve for the 9-3-3 pigment perchlorate is presented in Figure 9.

Thin layer chromatography on Silica gel G in ethyl ether, ethyl acetate, and methanol of the 9-3-3 pigment perchlorate and of prodigiosin showed that both pigments behaved identically in these solvents.

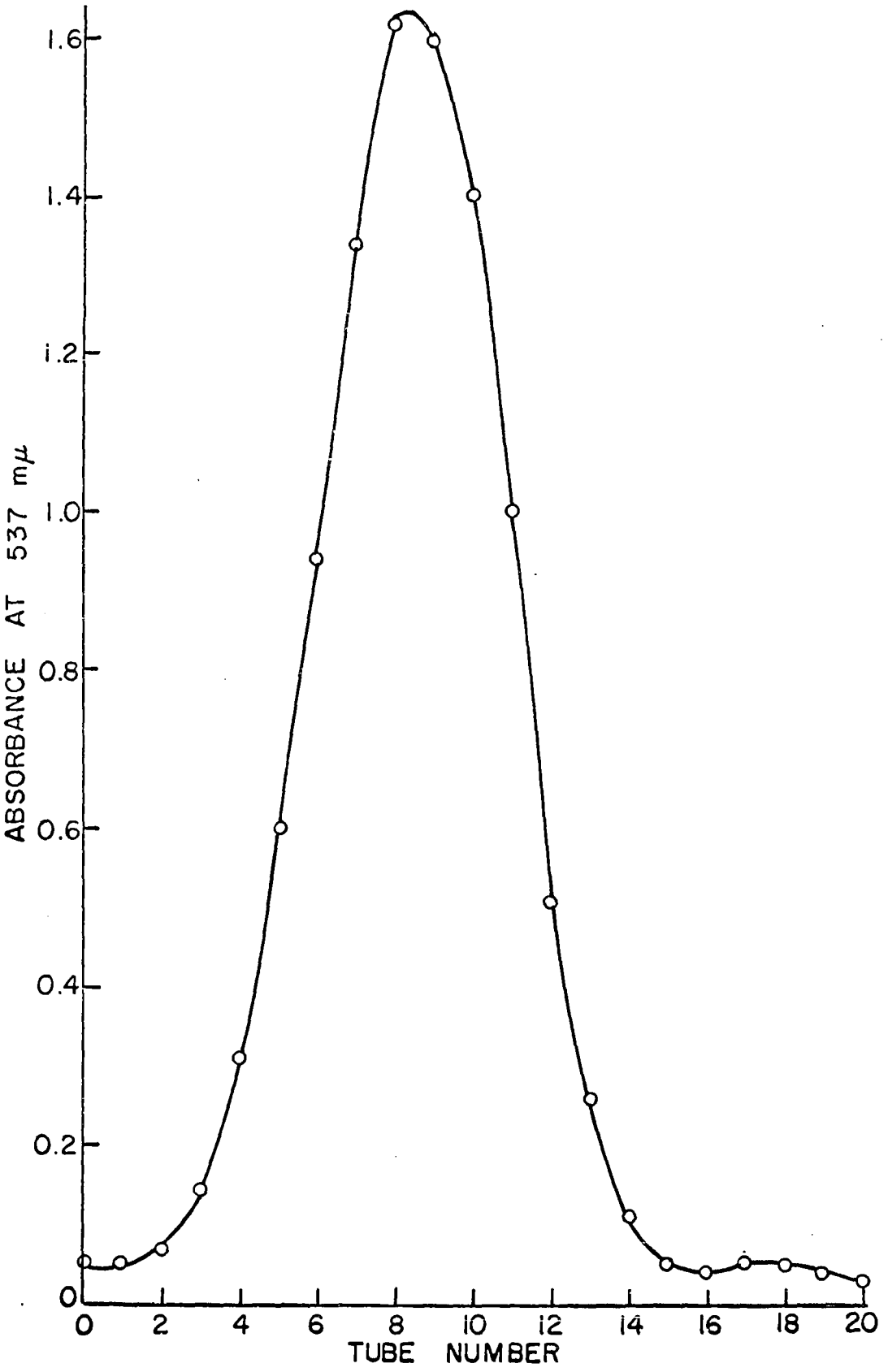
Elemental analysis of the pigment perchlorate yielded the following results: Calculated for $C_{20}H_{25}N_3O \cdot HClO_4$: C, 56.6; H, 6.13; N, 9.91. Found: C, 56.4; H, 6.49; N, 9.27.

Prodigiosin Analogs

General observations

It has already been mentioned that the final step in the biosynthesis of prodigiosin is enzymatic coupling of 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde with 2-methyl-3-amylpyrrole. Mutant 9-3-3 was shown to accumulate the final bipyrrole precursor and when fed 2-methyl-3-amylpyrrole could couple this monopyrrole to form prodigiosin. Wasserman observed that 2,4-dimethylpyrrole could substitute for the natural monopyrrole to produce an analog of prodigiosin, presumably differing from prodigiosin only in the nature of the alkyl substituents bound

Figure 9. Countercurrent distribution curve of 9-3-3 prodigiosin-like pigment in Skelly B, methyl-cellosolve, 0.01 M phosphate buffer, pH 7.2 (4:3:1)


ABSORBANCE AT 537 mμ
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
18
20
TUBE NUMBER

to the monopyrrole moiety of the molecule. It was found in this laboratory that kryptopyrrole could also substitute for the natural monopyrrole, in fact more efficiently than 2,4-dimethylpyrrole. Assuming similar molar absorptivities, kryptopyrrole was found to be incorporated about three times more efficiently than 2,4-dimethylpyrrole, based on pigment extract absorbance readings checked after equal times of incubation of monopyrrole. It was also found that 2-methylpyrrole could substitute for 2-methyl-3-amylpyrrole, but to a lesser extent than the other two monopyrroles. Pyrrole itself was observed not to be incorporated at all.

In the present study, the 9-3-3 strain was used as a source of bipyrrole and coupling enzyme. Analogs of prodigiosin were formed by the addition of alkylmonopyrroles other than 2-methyl-3-amylpyrrole. The analogs were isolated and their properties compared to those of authentic prodigiosin

Production of analogs

Strain 9-3-3 was grown in peptone-glycerol broth cultures. Williams' minus-phosphate broth was undesirable for the production of analogs of prodigiosin because strain 9-3-3 produces prodigiosin in this medium, as shown in the preceding section. The peptone-glycerol medium of Santer had the important advantage of producing no prodigiosin while still accumulating an amount of bipyrrole comparable to that produced in the Williams'

minus-phosphate medium. That strain 9-3-3 produced no prodigiosin in the peptone-glycerol medium was shown by the following experiment: Strain 9-3-3 peptone-glycerol broth cultures were grown for 48 hours at 28°. Pigment level in the 48-hour culture was checked by the method of Williams (29). To a 1-ml. aliquot of the 48-hour culture in a test tube was added 2 ml. of 1 N NaOH. The tube was placed in a boiling water bath for 10 minutes. One ml. of the mixture was then pipetted into a 10-ml. volumetric flask and 1 ml. of 1 N HCl was added. The flask was brought up to volume with distilled water. The absorbance of this sample was determined at 537 mμ, the acidic visible absorption maximum of prodigiosin, and at 655 mμ, to take into account the turbidity of the solution. The 655 mμ reading was subtracted from the 537 mμ reading to yield a measure of the prodigiosin level in the sample. This difference reading on 48-hour 9-3-3 peptone-glycerol broth cultures was negligible.

Analogs of prodigiosin were produced in the following way in a typical experiment: Twelve 2-l. Erlenmeyer flasks each containing 200 ml. of 0.5 per cent peptone-1.0 per cent glycerol broth wer autoclaved. The flasks were inoculated with a 2-ml. 24-hour tube inoculum which had in turn been inoculated with a needle from a 24-hour working culture. The tube inoculum was sometimes prepared from peptone-glycerol broth, but more often Williams' minus-phosphate broth was used, since this was

observed to support heavier cell growth than the peptone-glycerol medium. No prodigiosin was produced in 9-3-3 peptone-glycerol cultures utilizing Williams' minus-phosphate medium in the tube inoculum. The inoculated flasks were placed in an incubator shaker and shaken for 24 hours at 28° at a speed setting of 6. At the end of this time, 0.4 ml. of a solution containing 50 mg./ml. of monopyrrole in 95 per cent ethanol was pipetted into each flask. The cultures were grown for another 24 hours and the cells harvested by centrifugation. The isolation of analogs is described in the next two sections.

Isolation of the kryptopyrrole prodigiosin analog

The kryptopyrrole analog was isolated according to the procedures outlined for isolation of syntrophic pigments. After extraction of the pigment from the cellular mud using the procedure of Wrede, the hydrochloride and perchlorate salts were formed in the usual manner. The perchlorate salt was further purified by converting it to the free base and subjecting the free base to column chromatography on Super-cel. Development with 0.2 per cent methanol in Skelly B yielded a major red-orange fraction which was collected and evaporated to dryness. The pigment was redissolved in 95 per cent ethanol and heated. Dropwise addition of 5 per cent $HClO_4$ yielded the perchlorate salt in the form of tiny deep-blue needles. From the original perchlorate sample, the column-purified perchlorate salt was obtained in about 60 per cent yield. The crystalline product was washed several times with cold distilled

water and dried over $CaSO_4$ in a vacuum desiccator. Elemental analysis of the perchlorate salt yielded the following results: Calculated for $C_{18}H_{21}N_3O \cdot HClO_4$: C, 54.6; H, 5.57; N, 10.6. Found: C, 55.2; H, 5.82; N, 10.2. The molar absorptivity at the acidic visible absorption maximum of 537 mu was determined in acid ethanol. A solution containing $4.7^{-} \times 10^{-6}$ moles per liter gave an absorbance of 0.578 and was found to have a molar absorptivity of 12.2×10^4.

Isolation of the 2,4-dimethylpyrrole prodigiosin analog

The 2,4-dimethylpyrrole prodigiosin analog was purified in a somewhat different manner from the kryptopyrrole analog. This section describes several procedures followed to achieve purification of this analog.

In one experiment, pigment was extracted from the cellular mud and the hydrochloride salt formed in the usual manner. The yield of hydrochloride from 7200 ml. of 48-hour 9-3-3 peptone-glycerol culture (monopyrrole added at 24 hours) was 115 mg. This compared to a yield of 480 mg. of hydrochloride of the kryptopyrrole analog obtained from the same scale experiment. The hydrochloride was dissolved in 20 ml. of 95 per cent ethanol and 1 N NaOH added dropwise until the solution turned brown. Twenty ml. each of water and $CHCl_3$ were then added and the mixture shaken gently. The bottom layer was drawn off, dried over anhydrous Na_2SO_4, and concentrated to 2-3 ml. This sample was applied to the top of a

2 cm. x 15 cm. Super-cel column. Development with $CHCl_3$ yielded one major brown band with a leading red edge. This was collected and after concentrating, rechromatographed on another identical Super-cel column. The brown band from this column was evaporated to dryness. The pigment was redissolved in 95 per cent ethanol and the perchlorate salt crystallized in the usual way. The product was washed several times with cold distilled water and dried. Yield of perchlorage: 27 mg.

The perchlorate salt was converted to the free base and subjected to column chromatography on Super-cel. Development with $CHCl_3$ yielded a leading orange band followed by a deep purple band. The orange band was collected and evaporated to dryness yielding a pigment residue that had a bright green metallic sheen (observed also on prodigiosin and the krypto-pyrrole analog). Infrared analysis of the dried free base sample yielded a well defined spectrum. Two days later it was observed that the sample had lost its green metallic sheen. Elemental analysis of the free base yielded the following results: Calculated for $C_{16}H_{17}N_3O$: C, 71.9; H, 6.37; N, 15.7. Found: C, 71.3; H, 7.85; N, 11.1. The carbon analysis was in good agreement with the calculated value, but the values obtained for hydrogen and nitrogen were not.

In another experiment the hydrochloride was converted to the free base as described in the preceding paragraph and subjected to column chromatography on silicic acid:celite filter aid (2:1). Development with 5 per cent methanol in $CHCl_3$

yielded a leading red band. The major part of the sample remained near the top of the column and was deep purple. The red fraction was collected and evaporated to dryness. It was then redissolved in 95 per cent ethanol and the perchlorate salt crystallized in the usual way. Infrared analysis of the freshly formed salt gave a well defined spectrum. Problems were encountered in formation of the perchlorate salt of this analog even from column purified pigment fractions. It was observed that the salt did not crystallize as readily as did that of prodigiosin or the other analog. Also, after several days, it was only partially soluble in organic solvents in which it had originally been completely soluble. Another indication of the instability of this salt was seen on chromatography of the free base formed from the perchlorate salt by addition of base and water to an ethanolic solution of the salt, followed by extraction with $CHCl_3$. Chromatography of the dried and concentrated $CHCl_3$ solution on Super-cel employing either $CHCl_3$ or 0.2 per cent methanol in Skelly B always left a large purple section remaining near the top of the column.

One other procedure was followed to obtain a pure pigment component from the crude cellular extract. Cellular mud was extracted according to the procedure of Wrede to yield a crude Skelly B extract of the analog. This was dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was redissolved in ethyl acetate and subjected to column chromatography on

silicic acid:celite filter aid (1:1). Development with ethyl acetate yielded a leading deep-red band, followed in order by a lighter red band and a small yellow band. Left at the top of the column was a purple component. The major light red band was collected. Thin layer chromatography of this fraction on Silica gel G in ethyl acetate proved this fraction to be free of pigment impurities. The ethyl acetate fraction was concentrated to dryness and the pigment residue redissolved in 95 per cent ethanol. The pigment was converted to the free base by the addition of 1 N NaOH and after the addition of water, extracted into Skelly B. The Skelly B extracts were pooled, dried, and concentrated to 20-30 ml. Dry hydrogen chloride gas was bubbled through this solution under a nitrogen atmosphere, resulting in the formation of a red precipitate. The hydrochloride was collected by centrifugation, washed several times with Skelly B and dried. Thin layer chromatography of the hydrochloride indicated the salt to be free of pigment impurities, but infrared analysis proved the product to be impure. Elemental analysis was not in good agreement with the calculated formula.

Soda lime distillation of the kryptopyrrole prodigiosin analog

Soda lime distillation was carried out on a 15 mg. sample of the free base of the kryptopyrrole analog. The procedure used was identical to that followed for the distillation of

Table 4. Vapor phase chromatography of the kryptopyrrole soda lime distillate and authentic kryptopyrrole

Sample	Column temp.	Detector temp.	Injector temp.	Flow rate	Retention time (average of 3 runs)
Kryptopyrrole analog distillate	160°	230°	215°	100 ml. per min.	2.20 min.
Kryptopyrrole solution	160°	230°	215°	100 ml. per min.	2.14 min.
Mixture (distillate and kryptopyrrole)	160°	230°	215°	100 ml. per min.	2.12 min.

syntrophic pigments. The distillate was shown to be identical to authentic kryptopyrrole by both vapor phase and thin layer chromatography. Vapor phase chromatography data are presented in Table 4.

Comparison of prodigiosin analogs to prodigiosin

Ultraviolet-visible spectra of both analogs are presented in Figures 10 and 11. The spectra were determined in both basic and acidic ethanol. The acidic absorption maximum of the 2,4-dimethylpyrrole analog was 527 mμ, compared to a value of 537 mμ for prodigiosin. The basic visible absorption maximum was 465 mμ, compared to 475 mμ for prodigiosin. Both the acidic and basic spectra of the kryptopyrrole analog were identical to the spectra of prodigiosin.

Figure 10. Ultraviolet-visible spectrum of kryptopyrrole prodigiosin analog

——— Spectrum in 95 per cent ethanol 0.01 N in HCl

– – – Spectrum in 95 per cent ethanol 0.01 N in NaOH

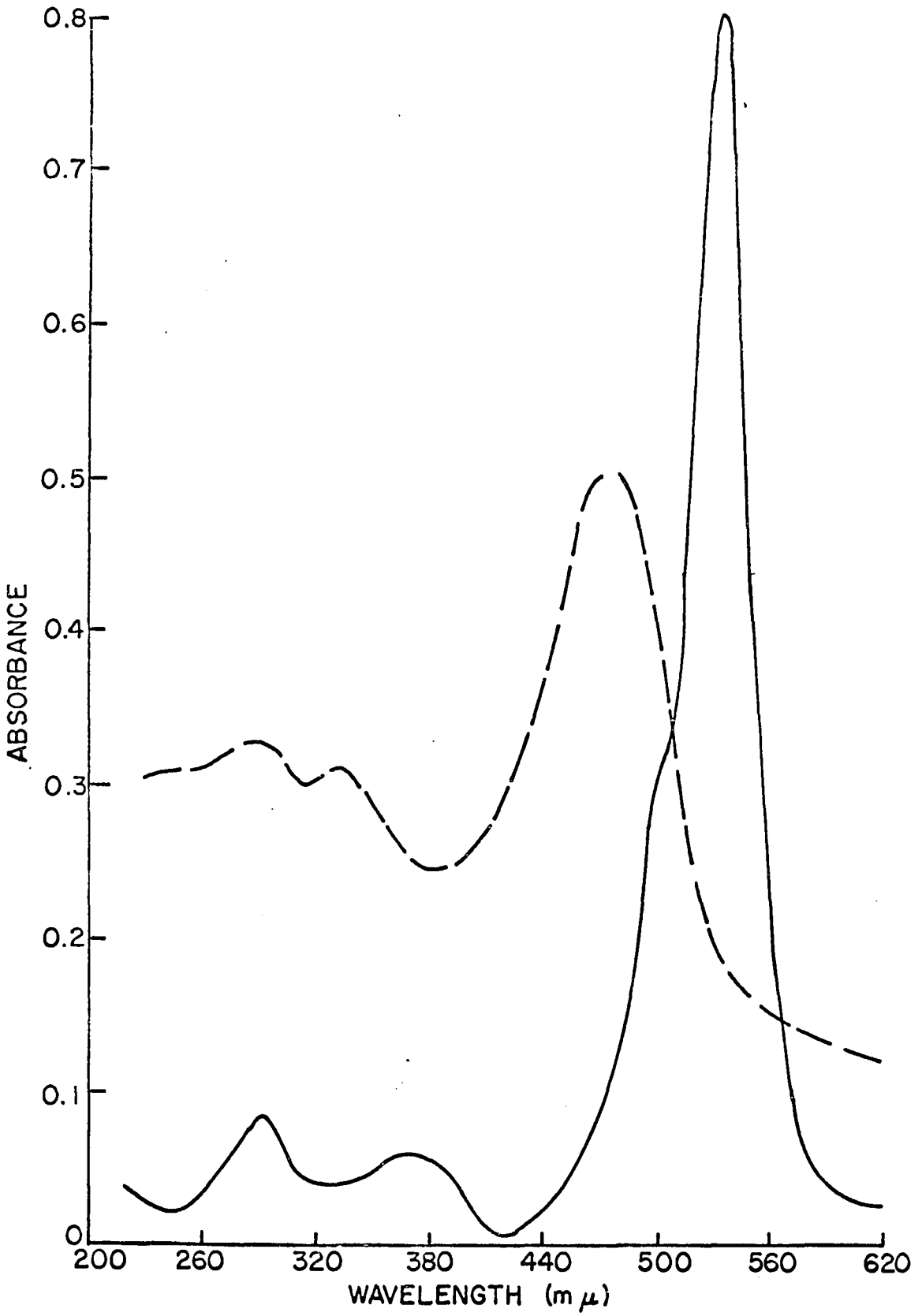
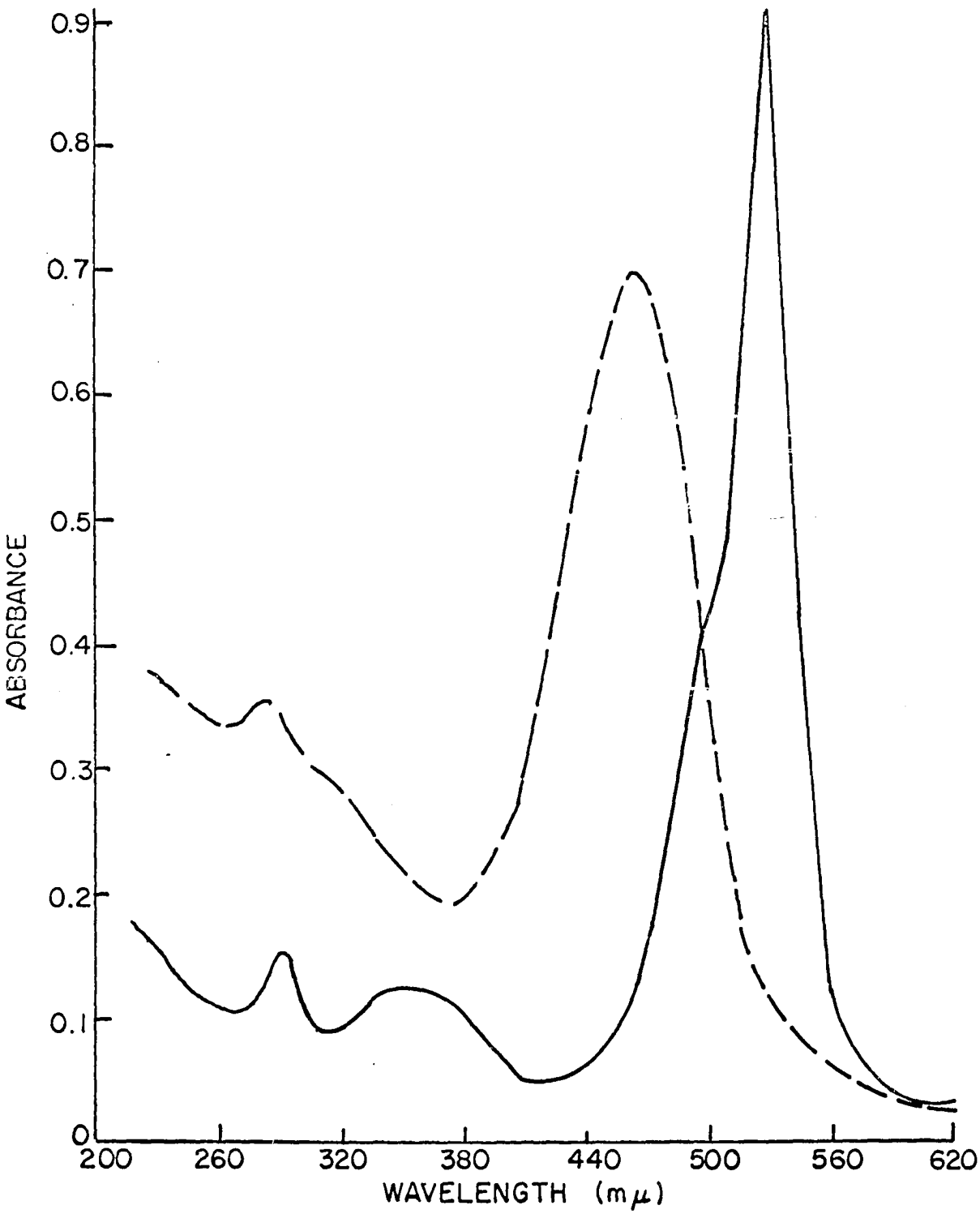

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
200
260
320
380
440
500
560
620
WAVELENGTH (mμ)

Figure 11. Ultraviolet-visible spectrum of 2,4-dimethylpyrrole prodigiosin analog

——— Spectrum in 95 per cent ethanol 0.01 N in HCl

– – – Spectrum in 95 per cent ethanol 0.01 N in NaOH


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
200
260
320
380
440
500
560
620
WAVELENGTH (mμ)

The infrared spectra of the free base of both analogs and of the free base of prodigiosin are presented in Figure 12. At 1400 cm^{-1} there was a peak in the prodigiosin spectrum which was absent in the spectra of both analogs. A peak at 1580 cm^{-1} in the prodigiosin spectrum was smaller in the kryptopyrrole spectrum and absent in the spectrum of the 2,4-dimethylpyrrole analog. The spectra also differ in several bands in the fingerprint region.

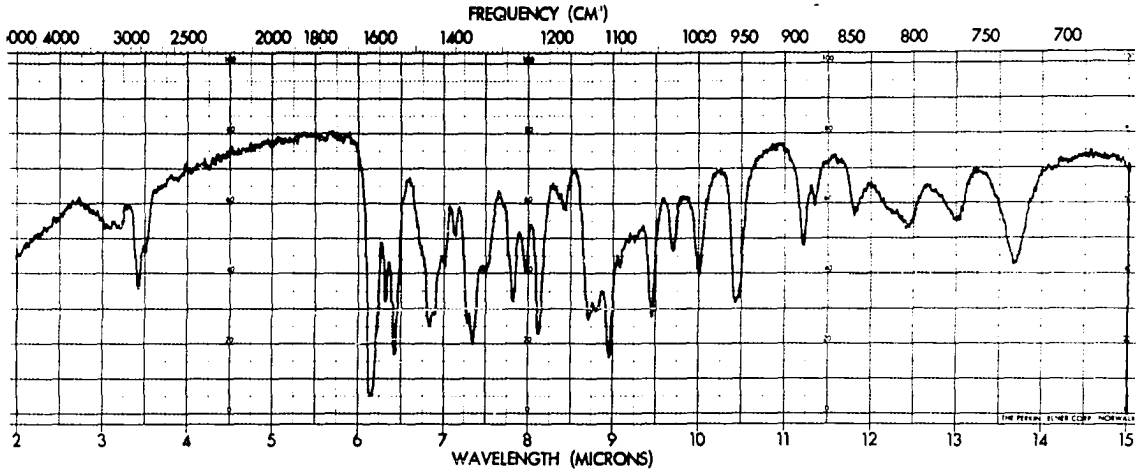
The n.m.r. spectra of the free base of both analogs and of prodigiosin are presented in Figure 13. The spectrum of prodigiosin had the most complex aliphatic region, with n.m.r. absorption at δ 0.83 (3H triplet, CH_3-amyl); 1.23 (6H complex absorption, $-(CH_2)_3-$); 1.70 (3H singlet, CH_3); 2.21 (2H triplet, CH_2-adj. to d.b.); 3.91 (3H singlet, $-OCH_3$); 6.05-6.80 (6H, aromatic C-H). The kryptopyrrole analog had n.m.r. absorption at δ 0.93 (3H triplet, CH_3-ethyl); 1.25 (2H singlet, impurity); 1.73 (3H singlet, CH_3); 2.09 (3H singlet, CH_3-superimposed on: 2.23 2H quadruplet, CH_2-ethyl); 3.91 (3H singlet, OCH_3); 6.05-6.90 (5H, aromatic C-H). The 2,4-dimethylpyrrole analog had n.m.r. absorption at δ 0.87-1.60 (proven impurity); 1.82 (3H singlet, CH_3); 2.14 (3H singlet, CH_3); 3.91 (3H singlet, OCH_3); 5.66-6.88 (6H, aromatic C-H: Note 5.66 1H singlet, attributable to aromatic C-H on monopyrrole β position).

Figure 12. Infrared spectra in KBr

Top. Prodigiosin free base

Center. Kryptopyrrole prodigiosin analog free base

Bottom. 2,4-dimethylpyrrole prodigiosin analog free base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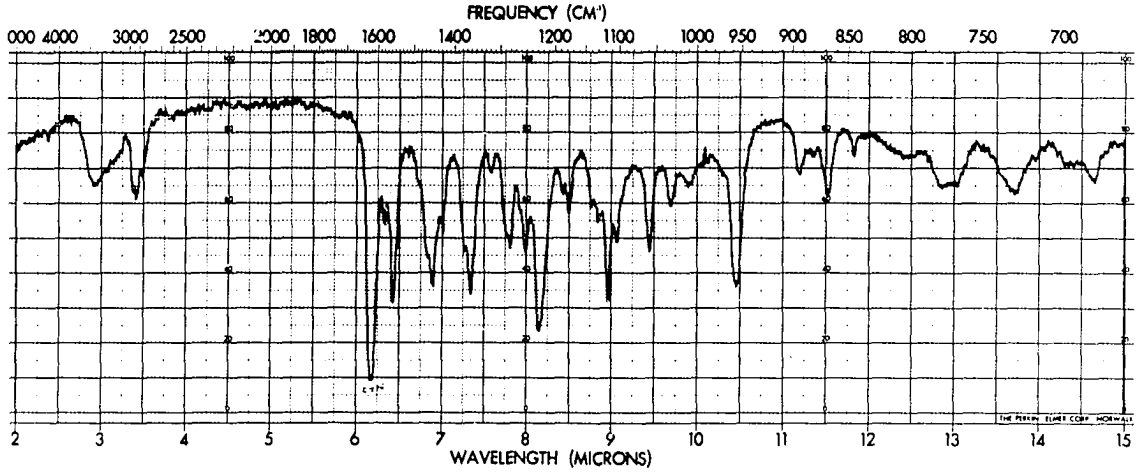
2 3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)


FREQUENCY (CM⁻¹)
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2 3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

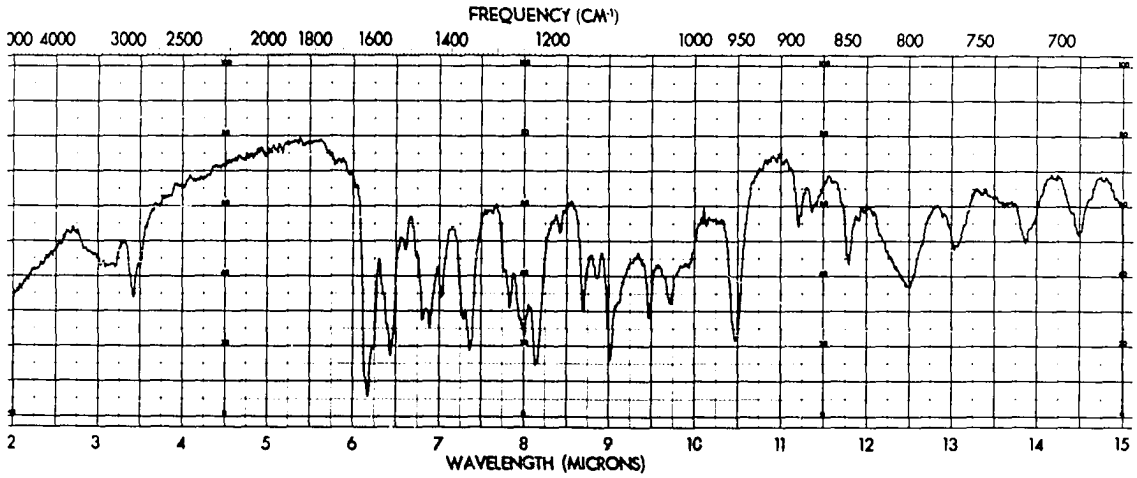
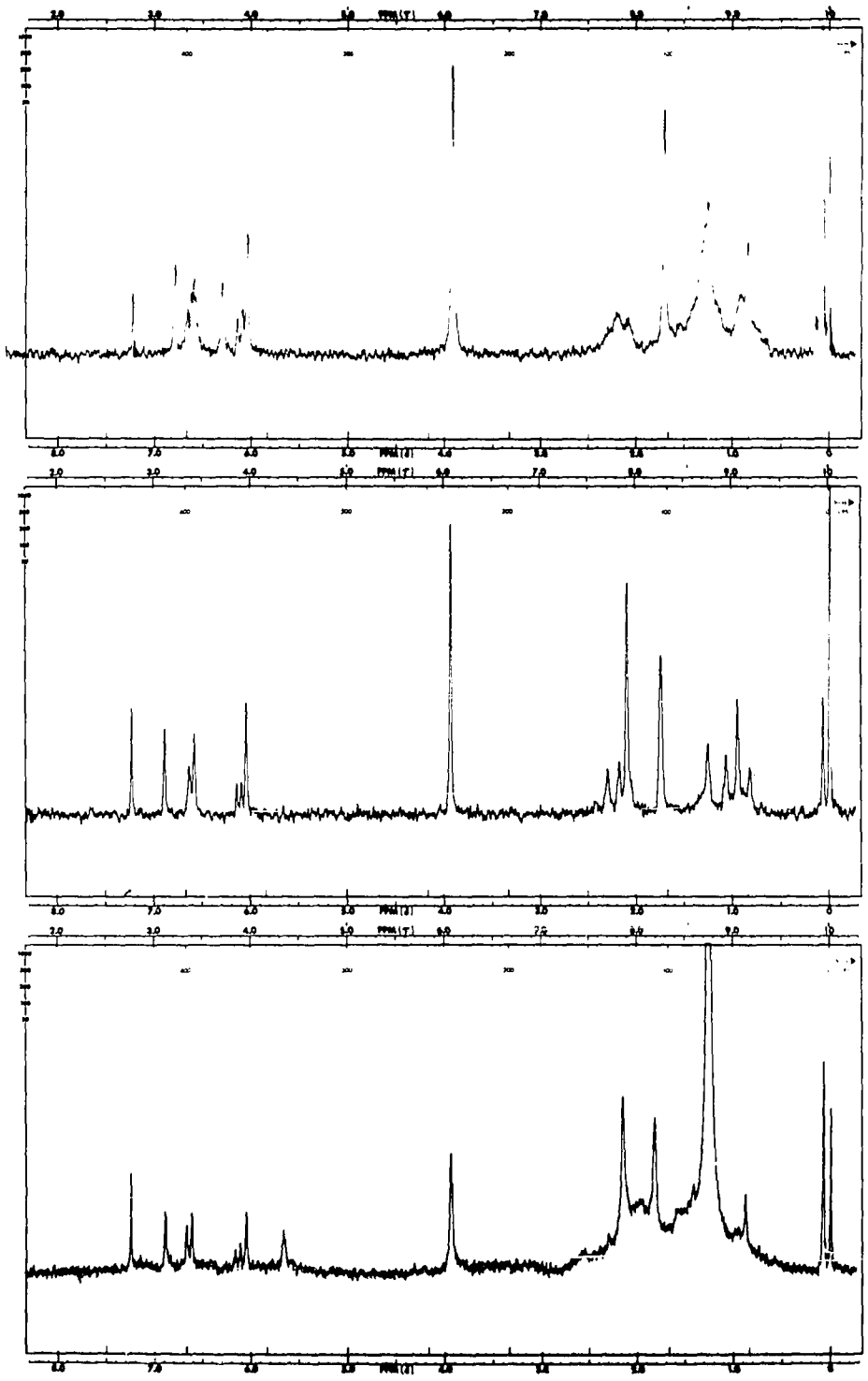

FREQUENCY (CM⁻¹)
000 4000 3000 2500 2000 1800 1600 1400 1200 1000 950 900 850 800 750 700
2 3 4 5 6 7 8 9 10 11 12 13 14 15
WAVELENGTH (MICRONS)

Figure 13. N.m.r. spectra at 60 Mc. in deuteriochloroform using tetramethylsilane (0.00 ppm.) as internal standard

Top. Prodigiosin

Center. Kryptopyrrole prodigiosin analog

Bottom. 2,4-dimethylpyrrole prodigiosin analog



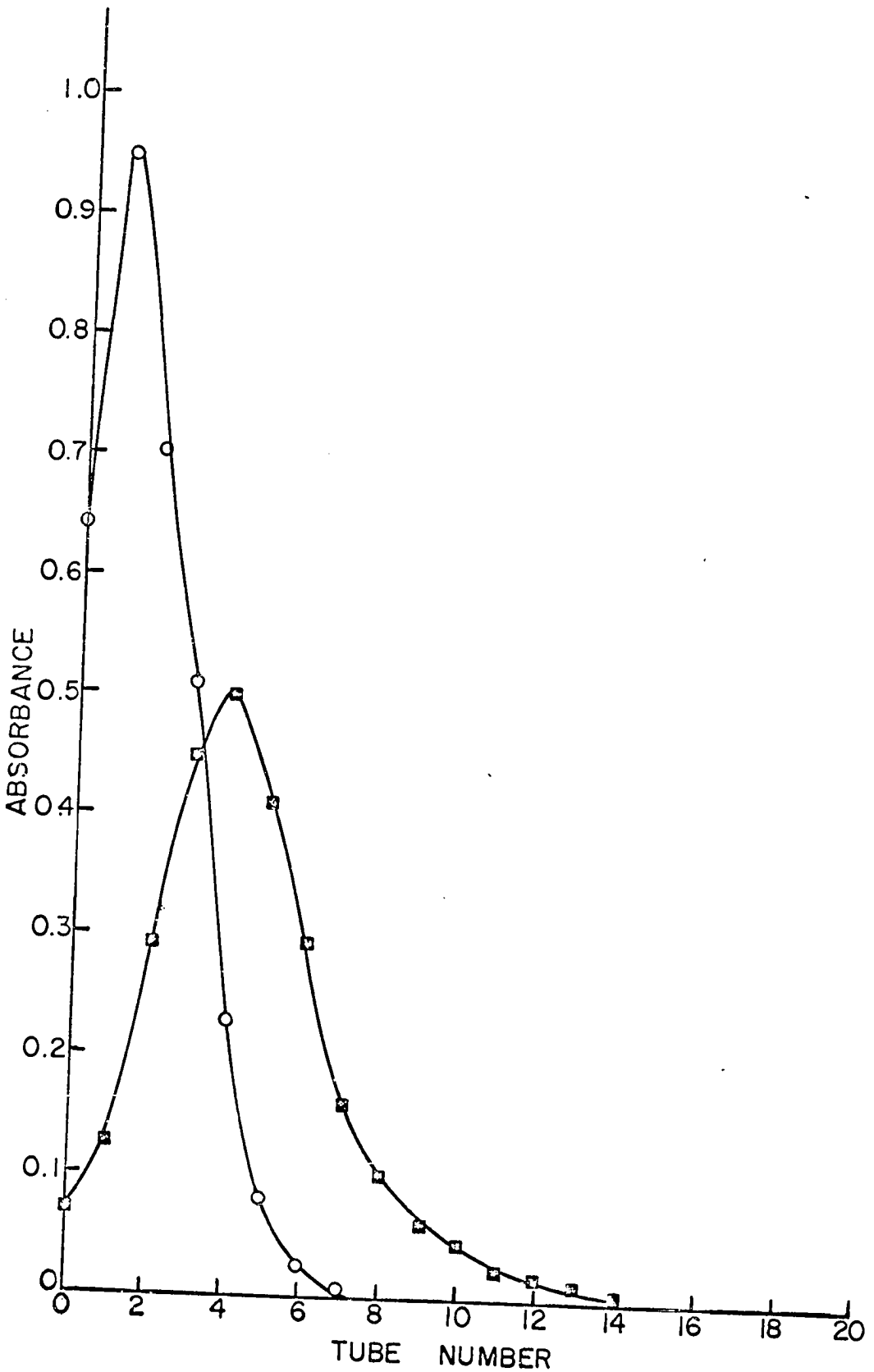
The prodigiosin analogs were subjected to countercurrent distribution. Twenty-transfer runs in the solvent system of Burgus described in the syntrophic pigment Experimental section yielded a partition coefficient K of 0.1 for the 2,4-dimethylpyrrole analog and 0.2 for the kryptopyrrole analog. The distribution curves for these runs are presented in Figure 14. Prodigiosin, as mentioned earlier, had a K of 0.8 in the same system. Separation of a mixture of the kryptopyrrole analog, the 2,4-dimethylpyrrole analog, and prodigiosin was attempted in the same solvent system of Burgus. The results of a 100-transfer run are presented in Figure 15. The 2,4-dimethylpyrrole analog had a K of 0.1 in the mixed run, the kryptopyrrole analog a value of 0.2, and prodigiosin, 0.7.

The analogs were compared to prodigiosin by thin layer chromatography. Migration of the analogs differed from that of prodigiosin in ethyl ether and ethyl acetate.

Figure 14. Countercurrent distribution curves in Skelly B, methylcellosolve, 0.01 M phosphate buffer, pH 7.2 (4:3:1)

– – – – Kryptopyrrole prodigiosin analog, absorbance readings at 537 mμ

– – – – 2,4-dimethylpyrrole prodigiosin analog, absorbance readings at 527 mμ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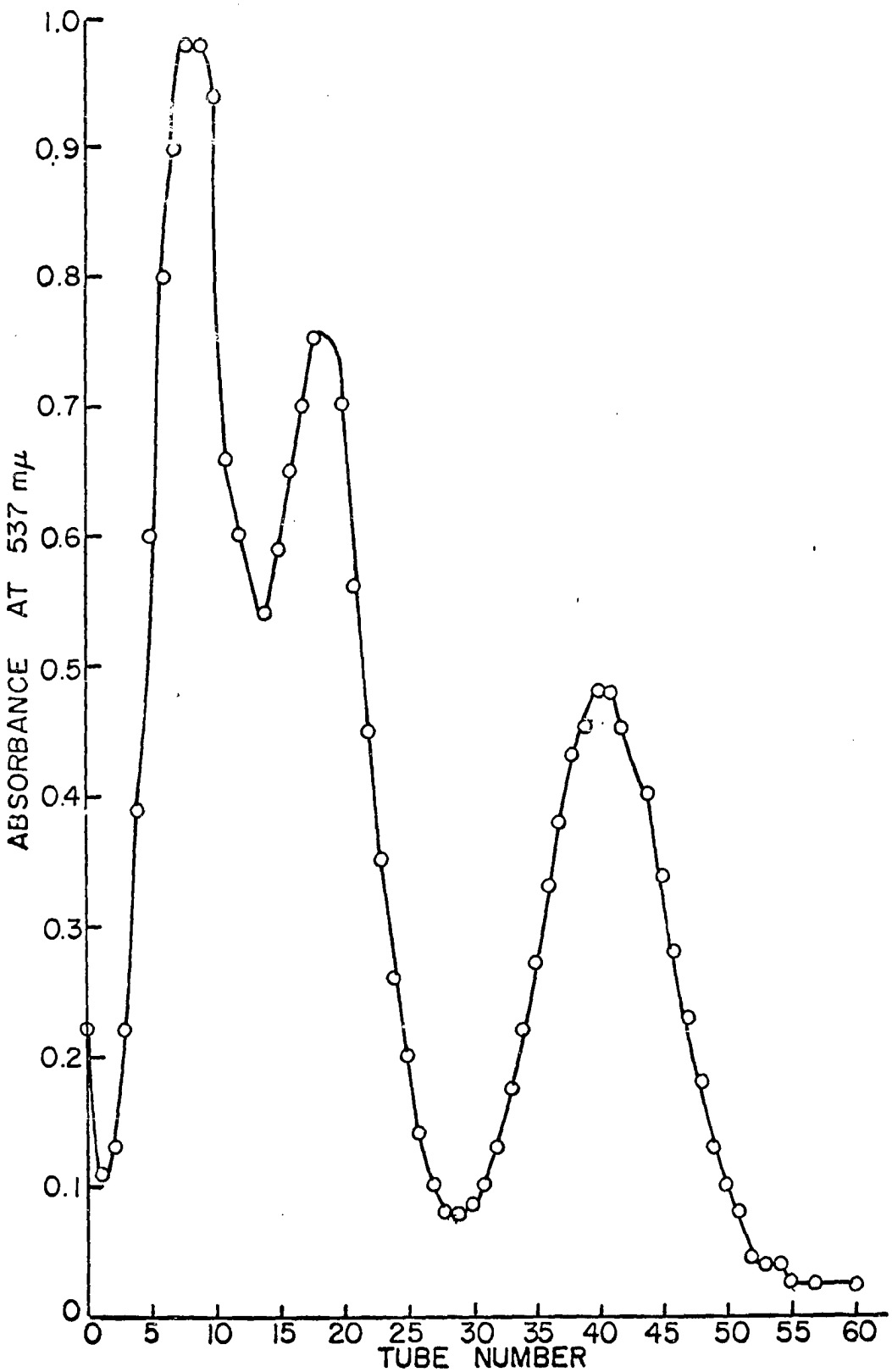
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
0
2
4
6
8
10
12
14
16
18
20
TUBE NUMBER

Figure 15. Countercurrent distribution curve of a mixture of kryptopyrrole prodigiosin analog, 2,4-dimethyl prodigiosin analog, and prodigiosin in Skelly B, methylcellosolve, and 0.01 M phosphate buffer, pH 7.2 (100 transfer run, absorbance readings on first 60 tubes) (4:3:1)


ABSORBANCE AT 537 mμ
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 5 10 15 20 25 30 35 40 45 50 55 60
TUBE NUMBER

DISCUSSION

Analogs of Prodigiosin

Studies on prodigiosin metabolism in Serratia marcescens and reports of the occurrence of prodigiosin and prodigiosin-like components in other species continue to appear in the literature, but with criteria for identification of pyrryldipyrrylmethene compounds which vary greatly from one laboratory to another. The availability of a Serratia mutant, strain 9-3-3, capable of synthesizing analogs of prodigiosin when supplied with exogenous alkylmonopyrroles, made it possible to test the ability of various analytical techniques to distinguish prodigiosin from closely related compounds. Two such analogs produced biosynthetically and purified sufficiently for comparison with prodigiosin were studied extensively by the present author.

It is not surprising that investigators often fail to report satisfactory chemical characterization of pyrrldipyrrylmethene compounds, since the inherent instability of pyrroles renders them difficult to purify. Sensitive to acid-catalyzed polymerization, and to photochemical degradations as well, they are seldom obtained free from impurities detectable by chromatography or countercurrent distribution. When rechromatography of a single sharp band always gives rise to additional minor bands, it is reasonable for an investigator to turn from

purification to analysis, recognizing that his analytical sample is not what he would like it to be. This frustrating situation has always been the case in this laboratory with prodigiosin and particularly with norprodigiosin (5); the present author encountered similar problems with the krypto-pyrrole analog and particularly with the 2,4-dimethylpyrrole analog.

Of course, for many applications, precise identification is not absolutely essential. The relatively simple means of identification to be used in a biological laboratory include melting point determinations and ultraviolet-visible spectra. Although the perchlorate salts of the pyrrldipyrrylmethene compounds form readily and are relatively easy to recrystallize, their melting or decomposition points are not very useful for identification. Castro (6) has described the difficulty of determining accurately the decomposition point of prodigiosin salts, reporting values varying as much as 10-15 degrees for highly purified samples of prodigiosin perchlorate. Similar difficulties were encountered in the present study with the perchlorate salts of the prodigiosin analogs.

The ultraviolet-visible spectrum of the C_{25} Streptomyces prodigiosin-like pigment has been reported (8) to be identical to that of prodigiosin. In this investigation the krypto-pyrrole analog was also found to have an ultraviolet-visible spectrum indistinguishable from that of prodigiosin. However, the 2,4-dimethylpyrrole analog was found to have a different

visible absorption maximum in both acid and base. Rapoport (19) noted slight differences in the spectrum of his synthetic 2-butyl-3-ethylpyrrole analog from that of prodigiosin. Thus it is clear that in some cases ultraviolet-visible spectra could be used to distinguish between two prodigiosin-like analogs, but also that a newly isolated pigment cannot be identified positively as prodigiosin on the basis of ultraviolet-visible spectra alone. The conclusion of Perry (18) that the pigment he isolated from a Streptomyces strain was prodigiosin solely on the basis of its ultraviolet-visible spectrum is unwarranted without supplementary analytical data.

Infrared analysis is generally more specific than ultraviolet-visible analysis. Careful examination of the infrared spectrum of prodigiosin showed it to be similar in many respects to that of each of the analogs prepared and also to the published spectrum of the C_{25} pigment (1), but with observable differences in each case. Noteworthy is a band at 730 cm^{-1} which is much stronger in the prodigiosin spectrum than in that of either analog. This band can be interpreted as being due to rocking deformation vibrations of the methylene groups in the amyl substituent. The band was also observed to be strong in the spectrum of the C_{25} pigment. Differences in the spectra of the two analogs and of prodigiosin were also observed at 1580 cm^{-1}, 1400 cm^{-1}, 1150 cm^{-1}, 1000 cm^{-1}, and at several other bands in the fingerprint region.

Nuclear magnetic resonance is sometimes a more powerful tool than infrared spectrophotometry. The n.m.r. absorption spectrum of prodigiosin was markedly different from that of either analog. The aliphatic region of the prodigiosin spectrum is complex, as is expected from the contribution due to the methylene amyl chain. Partial assignment of peaks has been made in the Experimental section.

Countercurrent distribution can be carried out with relatively inexpensive apparatus if many transfers are not necessary. It was found that a 100-transfer distribution is capable of good separation of prodigiosin and the two analogs. Thus, besides affording a characteristic partition coefficient which could be determined with only 20 transfers, countercurrent distribution was shown to be a useful tool for the separation of prodigiosin-like compounds. On a microgram scale, thin layer chromatography was also found capable of distinguishing prodigiosin from the two analogs in some solvents tested, but with only slight differences in Rf.

Degradative studies were originally used to characterize prodigiosin. The availability of vapor phase and thin layer chromatography to identify soda lime distillation products renders this a useful technique for identification. If the synthetic monopyrrole is available, the cleavage product can can be compared with it directly, if not, authentic prodigiosin can be subjected to soda lime distillation to provide 2-methyl--3-amylpyrrole as the major degradative product for comparison.

Thus in this investigation the syntrophic pigments were shown to give cleavage products identical to those obtained from prodigiosin. In the case of the kryptopyrrole analog it was possible to compare the cleavage products obtained from soda lime distillation to a sample of authentic kryptopyrrole; the distillate was shown to be identical to the authentic monopyrrole.

Thus, for applications in which positive chemical identification of pyrryldipyrrylmethene compounds is essential to avoid drawing unwarranted biological conclusions, studies with two biosynthetic analogs of prodigiosin indicate several useful criteria. Comparison of infrared spectra as well as ultraviolet-visible spectra is a sine qua non for studies of prodigiosin-like compounds, and requires only a few milligrams of purified product. Comparison of n.m.r. spectra is a valuable adjunct to comparison of infrared spectra, but suffers from the disadvantage of requiring larger amounts of sample. Perhaps mass spectral data would be even more useful if the equipment were available. Observation of behavior on column and thin layer chromatography, elemental analysis, determination of the partition coefficient by countercurrent distribution, and identification of fragments from soda lime distillation were all shown to provide useful supplementary information, but not in themselves to be definitive enough as a sole means of identification.

In addition to giving opportunity to test the validity of methods used for identification of prodigiosin, biosynthetic analogs of prodigiosin are of interest because of information they provide about the specificity of the coupling enzyme in the 9-3-3 system. The coupling enzyme has now been found to incorporate several alkylmonopyrroles other than the natural one, including kryptopyrrole, 2,4-dimethylpyrrole, and 2-methylpyrrole. Pyrro e and pyrrole-2-carboxaldehyde were observed not to be utilized. Some of the differences in rates of incorporation are perhaps due as much to ring-activation effects of the various substituents as to steric factors.

Syntrophic Pigments

Infrared and ultraviolet-visible analysis, as well as elemental analysis, countercurrent distribution, thin layer chromatography, and degradative studies established the fact that the pigments produced by the mutant pairs OF/H-462 and WCF/H-462 are both identical to prodigiosin. The sequence of steps blocked in the three mutants is thus established, the order being WCF → H-462 → OF. With the chemical confirmation of the location of H-462 in the sequence, investigation of the diffusible precursor or precursors elaborated by this strain takes on added significance. Work in the laboratory by Bascur de Medina (15) has produced some as yet uncharacterized Ehrlich-positive fractions from mutant H-462;

characterization of compounds might provide important clues to the current mystery of the formation of the pyrrole rings in the bipyrrole moiety of prodigiosin.

Pigment Production in Mutant 9-3-3

Although evidence indicates that the pyrrolidine ring of proline may be converted to the pyrrole ring of 2-methyl-3-amylpyrrole in Serratia marcescens (24), nothing is known about the origin of the two alkyl substituents. Hence the biosynthesis of the monopyrrole moiety of prodigiosin is almost as much a mystery at present as biosynthesis of the bipyrrole moiety. At least the three mutants mentioned above have been located in the bipyrrole pathway, but apparently few if any mutants in the monopyrrole pathway other than 9-3-3 exist. Studies on strain 9-3-3 are thus of great interest, and the finding by R. P. Williams that a purple pigment is formed by this normally nonpigmented strain under certain growth conditions is particularly significant. This purple pigment was shown by the present author to be easily resolvable into red and blue components which could be further purified, plus a large amount of apparently highly polymerized pyrrole-containing material. Similarity of the red pigment to prodigiosin was immediately obvious from chromatographic behavior of the pigment, and when the sensitive analytical techniques explored in this investigation were applied, the red component was shown to be identical to prodigiosin.

Normally strain 9-3-3 produces bipyrrole and coupling enzyme but is unable to form prodigiosin because of inability to synthesize 2-methyl-3-amylpyrrole; it thus "feeds" the three mutants in the bipyrrole pathway and at the same time is fed by them because all of them supply the monopyrrole. It was somewhat surprising to find that in a medium deficient in phosphate salts this strain has the ability to synthesize 2-methyl-3-amylpyrrole, as evidenced by synthesis of prodigiosin under these conditions. This investigation has thus shown that phosphate has an inhibitory effect on some step or steps in the biosynthesis of the monopyrrole. The nature of this inhibition has not yet been studied, but is clearly of interest.

Further work is also under way in this laboratory to elucidate the structure of the blue fraction produced under phosphate deficient conditions. Without a sequence of mutants in the monopyrrole pathway it would be impossible to search for "feeding factors" or precursors of the monopyrrole moiety of prodigiosin. Furthermore, production of the complete bipyrrole moiety of strain 9-3-3 complicates a search for pyrrole-containing precursors related to the monopyrrole. However, presence of the bipyrrole precursor can serve as a trapping agent for monopyrroles capable of coupling to form pyrryldipyrrylmethene compounds which can be isolated as more or less stable pigments. Thus in this investigation the identification of prodigiosin has established that the enzymes for

synthesizing 2-methyl-3-amylpyrrole are present in strain 9-3-3 when grown on a phosphate deficient medium. The blue pigment appears from solubility and chromatographic behavior at present to be quite different from prodigiosin; however, if it should turn out to be a pyrryldipyrrlmethene compound itself, another step forward will have been taken in elucidating the biosynthetic pathway to 2-methyl-3-amylpyrrole in Serratia marcescens.

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